

# ROSSI'S

# Principles of Transfusion Medicine

FOURTH EDITION

Toby L. Simon | Edward L. Snyder  
Bjarte G. Solheim | Christopher P. Stowell  
Ronald G. Strauss | Marian Petrides



WILEY-BLACKWELL

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EDITED BY

Toby L. Simon

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 **WILEY-BLACKWELL**

A John Wiley & Sons, Ltd., Publication



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*Editorial offices:* 9600 Garsington Road, Oxford, OX4 2DQ, UK  
The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK  
111 River Street, Hoboken, NJ 07030-5774, USA

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#### *Library of Congress Cataloging-in-Publication Data*

Rossi's principles of transfusion medicine / edited by Toby L. Simon . . . [et al.]. – 4th ed.  
p. ; cm.

Includes bibliographical references and index.

ISBN 978-1-4051-7588-3

1. Blood—Transfusion. I. Rossi, Ennio Claudio, 1931- II. Simon, Toby L. III. Title: Principles of transfusion medicine.

[DNLM: 1. Blood Transfusion. 2. Blood Banks—organization & administration. 3. Blood Grouping and Crossmatching. WB 356 R835 2009]

RM171.P75 2009

615'.39—dc22

2008038577

ISBN: 978-1-4051-7588-3

A catalogue record for this book is available from the British Library.

Set in 9.25/12 Minion Regular by Charon Tech Pvt Ltd.

Printed & bound in Singapore by Markono Print Media Pte Ltd.

1 2009



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# Preface

More than 6 years have elapsed since the third edition of *Rossi's Principles of Transfusion Medicine* was published. During that time, the field has continued to be dynamic and many advances have been made. Molecular techniques are increasingly utilized; measurements of cell kinetics have been further refined; new treatments for bleeding disorders have been developed; progenitor cell transplantation protocols have matured; hemovigilance efforts have become more organized globally; pathogen inactivation and apheresis techniques have advanced; new treatments have emerged for iron overload; gene therapy and regenerative medicine are having an impact on the field; and regulation has become more complex in a global environment.

As expected, some controversies remain unresolved. For example, the effect of red cell age on clinical outcome and whether transfusion itself affects patient recovery are at the center of recent articles that commanded attention from both professionals and the public. At the same time, pharmacologic agents intended to reduce the need for transfusion have also become subjects of controversy. Whether recombinant human erythropoietin has deleterious effects on patients, the potential for cell-free hemoglobin solutions to cause more harm than good, and the balance between efficacy and toxicity of recombinant Factor VIIa are being discussed at the national level of health-care decision-making.

This edition provides updated comprehensive assessments of the subjects that remain important to the daily work of those who practice transfusion medicine today. The contents include information on controversies that are still percolating, as well as those new developments that have been widely adopted in practice. This volume also features those scientific areas that represent the frontiers of transfusion medicine such as cellular and gene therapy and regenerative medicine.

Section I reviews the preparation and use of components and derivatives of blood used in clinical practice. Section II provides information and approaches to the major clinical issues related to transfusion in medical, obstetric, pediatric, oncology, and surgery patients, including transplant recipients. Section III focuses

attention on both donor and patient apheresis. Section IV introduces the major concerns related to hazards of transfusion and covers the infectious and noninfectious risks in separate chapters. Section V covers the broad issues related to cells and tissue therapy in our field. Section VI contains an overview of the logistic and regulatory aspects of the profession.

The fourth edition is an example of both continuity and change. We have retained the general organizational plan of the prior edition. The comprehensive scope has been retained with emphasis on the scientific and clinical over the technical. Our objective is to create the best single reference for the professional who is managing transfusion issues both at the bedside and at the interface of the laboratory and the clinic. Yet at the same time, the volume is intended to provide a helpful resource for trainees.

New chapters that have either combined material from several chapters in prior editions or ventured into new areas include cell kinetics, obstetric practice, cord blood, transfusion alternatives, regenerative medicine, hemovigilance, iron overload, and transplant organization and regulation. Once again, case studies have been included to enhance the educational aspects, but with this edition they are in an electronic format. In addition to the CD-ROM containing the case studies a second CD-ROM contains the 66 book chapters as searchable pdf files.

Four of the editors from the last edition have returned along with many of the expert chapter authors. This edition also welcomes new chapter contributors with fresh approaches and two new editors—Bjarte G. Solheim, who has led the effort to include more international perspectives, and Marian Petrides, who developed the software for, and managed the transition to, the interactive case study format. Joining the team effort for this edition are publishing staff at AABB Press and Wiley-Blackwell.

In a work of this magnitude, there are many to whom we owe words of appreciation. Clearly, thanks are due to the authors for their superb chapters, AABB Press staff for their expertise and assistance, and personnel at Wiley-Blackwell for their professional and collegial efforts on behalf of the book. Just as

important, the editors thank our families, colleagues, employers, teachers, and students for the support that sustained us throughout the development process.

All of us have chosen to work in this field because of our commitment to improving and saving the lives of those in our communities who can benefit from transfusion therapies. Some have used the analogy of those who work backstage in a theatrical production; the actors take the bows but the play can go on only with the contributions of the unseen workers behind the curtains. When the surgeons, oncologists, pediatricians, obstetricians, intensive care specialists, other physicians, and nurses are successful in treating the patients, those of us in transfusion

medicine and related therapies can take satisfaction in the work we do. Our hope is that this book will help support those who can make a difference in transfusion medicine and serve as an example of the teamwork so urgently needed to move all of health care forward for the benefit of the global community.

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# 1

## Transfusion in the New Millennium

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Prehistoric man left drawings of himself pierced by arrows.<sup>1</sup> This means he was as aware of blood as he was of his own limbs. The flint implements he used as tools and weapons distinguished him from other creatures and contributed to the violence of his era. As he hunted food and fought enemies, he observed bleeding and the properties of blood. A cut, received or inflicted, yielded a vivid red color. If the cut was shallow, there was little blood. But if the cut was deep, a red torrent flowing from the stricken victim quickly led to death, with shed blood congealed and darkening in the sun. Fatal hemorrhage was commonplace. Nonetheless, the sight must have been fearful and possibly existential as life flowed red out of the body of an enemy or a wounded animal.<sup>2</sup> It is no wonder, then, that at the dawn of recorded history, blood was already celebrated in religious rites and rituals as a life-giving force.

The cultural expressions of primitive and ancient societies, though separated by time or space, can be strikingly similar. Whether these expressions emerged independently or were diffused about the world by unknown voyagers will probably always remain clouded in mystery.<sup>2</sup> Nonetheless, there is a common thread in the ancient rituals that celebrate blood as a mystical vital principle. In Leviticus 17:11, “the life of the flesh is in the blood,” and the Chinese Neiching (circa 1000 BC) claims the blood contains the soul.<sup>2</sup> Pre-Columbian North American Indians bled their bodies “of its greatest power” as self-punishment,<sup>3</sup> Egyptians took blood baths as a recuperative measure, and Romans drank the blood of fallen gladiators in an effort to cure epilepsy.<sup>4</sup> The Romans also practiced a ceremony called taurobolium—a blood bath for spiritual restoration. A citizen seeking spiritual rebirth descended into a pit or fossa sanguinis. Above him on a platform, a priest sacrificed a bull, and the animal’s blood cascaded down in a shower upon the beneficiary. Then, in a powerful visual image, the subject emerged up from the other end of the pit, covered with blood and reborn.<sup>1</sup>

The legend of Medea and Aeson taken from Ovid’s *Metamorphoses* and quoted in Bulfinch’s *Mythology*<sup>5</sup> also ascribed rejuvenating powers to blood. Jason asked Medea to “take some years off his life and add them to those of his father Aeson.” Medea, however, pursued an alternative course. She prepared a cauldron with the blood of a sacrificed black sheep. To this, she added magic herbs, hoarfrost gathered by moonlight, the entrails of a wolf, and many other things “without a name.” The boiling cauldron was stirred with a withered olive branch, which became green and full of leaves and young olives when it was withdrawn. Seeing that all was ready,

Medea cut the throat of the old man and let out all his blood, and poured into his mouth and into his wound the juices of her cauldron. As soon as he had imbibed them, his hair and beard laid by their whiteness and assumed the blackness of youth; his paleness and emaciation were gone; his veins were full of blood, his limbs of vigour and robustness. Aeson is amazed at himself and remembers that such as he now is, he was in his youthful days, 40 years before. This legend seems to echo in the apocryphal story of Pope Innocent VIII, who is said to have received the blood of three young boys in 1492 while on his deathbed. As the story goes, a physician attempted to save the pope’s life by using blood drawn from three boys 10 years of age, all of whom died soon thereafter. Some 19th-century versions of this tale suggest the blood was transfused. However, earlier renditions more plausibly suggest that the blood was intended for a potion to be taken by mouth. In any event, there is no evidence the pope actually received any blood in any form.<sup>6,7</sup>

The folklore that flowed with blood was not accompanied by a great deal of accurate information. The ancient Greeks believed that blood formed in the heart and passed through the veins to the rest of the body, where it was consumed. Arteries were part of an independent system transporting air from the lungs. Although Erasistratos (circa 270 BC) had imagined the heart as a pump, his idea was ahead of its time. As long as veins and arteries were dead-end channels transporting blood and air, there was little need for a pump in the system. Although Galen (131-201 AD) finally proved that arteries contain blood, communication with

the venous system was not suspected. Blood, formed in the liver, merely passed through the blood vessels and heart on its way to the periphery.<sup>1</sup> These teachings remained in place for 1400 years until they were swept away in 1628 by Harvey's discovery of the circulation.

The realization that blood moved in a circulating stream opened the way to experiments on vascular infusion. In 1642 George von Wahrendorff injected wine<sup>8</sup>—and in 1656 Christopher Wren and Robert Boyle injected opium and other drugs<sup>9</sup>—intravenously into dogs. The latter studies, performed at Oxford, were the inspiration for Richard Lower's experiments in animal transfusion.

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## The First Animal Transfusion

Richard Lower (1631-1691) was a student at Oxford when Christopher Wren and Robert Boyle began their experiments on infusion. In due course, Lower joined their scientific group and studied the intravenous injection of opiates, emetics, and other substances into living animals.<sup>10</sup> In time, the transfusion of blood itself became the objective. The announcement of the first successful transfusion, performed by Richard Lower at Oxford in February 1665, was published on November 19, 1666, in the *Philosophical Transactions of the Royal Society Transactions (Transactions)* in a short notation titled, "The Success of the Experiment of Transfusing the Blood of One Animal into Another."<sup>11</sup> The entire notation is as follows<sup>11</sup>:

This experiment, hitherto look'd upon to be of an almost insurmountable difficulty, hath been of late very successfully perform'd not only at Oxford, by the directions of that expert anatomist Dr. Lower, but also in London, by order of the R. Society, at their publick meeting in Gresham Colledge: the Description of the particulars whereof, and the Method of Operation is referred to the next opportunity.

The December 17, 1666 issue of the *Transactions* contained the full description as promised.<sup>12</sup> It was taken from a letter<sup>13</sup> written by Lower to Robert Boyle on July 6, 1666, in which Lower described direct transfusion from the carotid artery of one dog to the jugular vein of another. After describing the insertion of quills into the blood vessels of the donor and recipient dogs, Lower wrote<sup>13</sup>:

When you have done this you may lay the dogs on their side and fasten them densely together as best you may to insure the connection of the two quills. Quickly tighten the noose around the neck of the receiving animal as in venasection, or at all events compress the vein on the opposite side of the neck with your finger, then take out the stopper and open the upper jugular quill so that while the foreign blood is flowing into the lower quill, the animal's own blood flows out from the upper into suitable receptacles—until at last the second animal, amid howls, faintings, and spasms, finally loses its life together with its vital fluid.

When the tragedy is over, take both quills out of the jugular vein of the surviving animal, tie tightly with the former slipknots,

and divide the vein. After the vessel has been divided, sew up the skin, slacken the cords binding the dog, and let it jump down from the table. It shakes itself a little, as though aroused from sleep, and runs away lively and strong, more active and vigorous perhaps, with the blood of its fellow than its own.

These studies inevitably led to the transfusion of animal blood to humans. In England, this occurred on November 23, 1667, when Lower and Edmund King transfused sheep blood into a man named Arthur Coga.<sup>14</sup> Described by Samuel Pepys as "a little frantic," Coga was paid 20 shillings to accept this transfusion, with the expectation that it might have a beneficial "cooling" effect. One week later, Coga appeared before the society and claimed to be a new man, although Pepys concluded he was "cracked a little in the head."<sup>15</sup> However, this was not the first transfusion performed in a human. The credit for that accomplishment belongs to Jean-Baptiste Denis (1635-1704), who had performed the first human transfusion several months earlier in Paris.

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## The First Animal-to-Human Transfusion

The founding of the Royal Society in London in 1662 was followed in 1666 by the establishment of the Academie des Sciences in Paris under the patronage of King Louis XIV. The new Academie reviewed the English reports on transfusion with great interest. Denis probably read of Lower's experiments in the *Journal des Savants* on January 31, 1667, and he began his own studies approximately 1 month later.<sup>15,16</sup> The first human transfusion was then performed on June 15, 1667, when Denis administered the blood of a lamb to a 15-year-old boy (Fig 1-1).

Although discovery of the circulation had suggested the idea of transfusion, indications for the procedure remained unformed. Transfusion was still thought to alter behavior and possibly achieve rejuvenation. The blood of young dogs made old dogs seem frisky; the blood of lions was proposed as a cure for cowardice; and 5 months later, Arthur Coga would receive a transfusion of sheep blood because of its presumed "cooling" effect. Denis used animal blood for transfusion because he thought it was "less full of impurities"<sup>17</sup>:

Sadness, Envy, Anger, Melancholy, Disquiet and generally all the Passions, are as so many causes which trouble the life of man, and corrupt the whole substance of the blood: Whereas the life of Brutes is much more regular, and less subject to all these miseries.

It is thus ironic that the symptoms of the first transfusion recipient may have been explained in part by profound anemia; the single transfusion of lamb blood may have produced temporary amelioration owing to increased oxygen transport. Denis described the case as follows<sup>17</sup>:

On the 15 of this Moneth, we hapned upon a Youth aged between 15 and 16 years, who had for above two moneths bin tormented with a contumacious and violent fever, which obliged his Physitians to bleed him 20 times, in order to assuage the excessive heat.

Before this disease, he was not observed to be of a lumpish dull spirit, his memory was happy enough, and he seem'd chearful and nimble enough in body; but since the violence of this fever, his wit seem'd wholly sunk, his memory perfectly lost, and his body so heavy and drowsie that he was not fit for anything. I beheld him fall asleep as he sat at dinner, as he was eating his Breakfast, and in all occurrences where men seem most unlikely to sleep. If he went to bed at nine of the clock in the Evening, he needed to be wakened several times before he could be got to rise by nine the next morning, and he pass'd the rest of the day in an incredible stupidity.

I attributed all these changes to the great evacuations of blood, the Physitians had been oblig'd to make for saving his life.

Three ounces of the boy's blood were exchanged for 9 ounces of lamb arterial blood. Several hours later the boy arose, and "for the rest of the day, he spent it with much more liveliness than ordinary." Thus the first human transfusion, which was heterologous, was accomplished without any evident unfavorable effect.

This report stimulated a firestorm of controversy over priority of discovery.<sup>18,19</sup> The letter by Denis was published in the *Transactions* on July 22, 1667, while the editor, Henry Oldenburg, was imprisoned in the Tower of London. Oldenburg, following some critical comments concerning the Anglo-Dutch War then in progress (1665-1667), had been arrested under a warrant issued June 20, 1667. After his release 2 months later, Oldenburg returned to his editorial post and found the letter published in his absence. He took offense at Denis's opening statement, which claimed that the French had conceived of transfusion "about ten years agoe, in the illustrious Society of Virtuosi . . ." (Fig 1-1). This seemed to deny the English contributions to the field. Oldenburg cited these omissions in an issue of the *Transactions* published September 23, 1667, "for the Months of July, August, and September." By numbering this issue 27 and beginning pagination with 489, Oldenburg attempted to suppress the letter by Denis.<sup>18</sup> However, as is evident, this did not ultimately succeed. Nonetheless, subsequent events created even greater difficulties for Denis.

Although the first two subjects who underwent transfusion by Denis were not adversely affected, the third and fourth recipients died. The death of the third subject was easily attributable to other causes. However, the fourth case initiated a sequence of events that put an end to transfusion for 150 years.

Anthony du Mauroy was a 34-year-old man who suffered from intermittent bouts of maniacal behavior. On December 19, 1667, Denis and his assistant Paul Emmerez removed 10 ounces of the man's blood and replaced it with 5 or 6 ounces of blood from the femoral artery of a calf. Failing to note any apparent improvement, they repeated the transfusion 2 days later. After the second transfusion, du Mauroy experienced a classic transfusion reaction<sup>20</sup>:

His pulse rose presently, and soon after we observ'd a plentiful sweat over all his face. His pulse varied extremely at this instant, and he complain'd of great pains in his kidneys and that he was not well in his stomach.

(489) *Numb. 27.*

# A LETTER

*Concerning a new way of curing sundry diseases by Transfusion of Blood, Written to Monsieur de MONTMOR, Counsellor to the French King, and Master of Requests.*

By J: DENIS *Professor of Philosophy, and the Mathematicks.*

Munday July 22. 1667.

SIR,



THE project of causing the Blood of a healthy animal to passe into the veins of one diseased, having been conceived about ten years agoe, in the illustrious Society of Virtuosi which assembles at your house, and your goodness having received M. Emmerez, & my self, very favorably at such times as we have presum'd to entertain you either with discourse concerning it, or the sight of some not inconsiderable effects of it: You will not think it strange that I now take the liberty of troubling you with this Letter, and design to inform you fully of what pursuances and successes we have made in this Operation; wherein you are justly intitled to a greater share than any other, considering that it was first spoken of in your *Academy*, & that the Publick is beholding to you for this as well as for many other discoveries, for the benefits & advantages it shall reap from the same.

But that I may give you the reasons of our procedure & convince

Ccc

Figure 1-1. The first human transfusion.<sup>17</sup>

Du Mauroy fell asleep at about 10 o'clock in the evening. He awoke the following morning and "made a great glass full of urine, of a colour as black, as if it had been mixed with the soot of chimneys."<sup>20</sup> Two months later, the patient again became maniacal, and his wife again sought transfusion therapy. Denis was reluctant but finally gave in to her urgings. However, the transfusion could not be accomplished, and du Mauroy died the next evening.

The physicians of Paris strongly disapproved of the experiments in transfusion. Three of them approached du Mauroy's widow and encouraged her to lodge a malpractice complaint against Denis. She instead went to Denis and attempted to extort money from him in return for her silence. Denis refused and filed a complaint before the Lieutenant in Criminal Causes. During the subsequent hearing, evidence was introduced to indicate that Madame du Mauroy had poisoned her husband with arsenic. In a judgment handed down at the Chatelet in Paris on April 17, 1668, Denis was exonerated, and the woman was held for trial. The court also stipulated "that for the future no Transfusion should

be made upon any Human Body but by the approbation of the Physicians of the Parisian Faculty.”<sup>21</sup> At this point, transfusion research went into decline, and within 10 years it was prohibited in both France and England.

## The Beginnings of Modern Transfusion

After the edict that ended transfusion in the 17th century, the technique lay dormant for 150 years. Stimulated by earlier experiments by Leacock, transfusion was “resuscitated” and placed on a rational basis by James Blundell (1790-1877), a London obstetrician who had received his medical degree from the University of Edinburgh.<sup>22</sup> Soon after graduation, Blundell accepted a post in physiology and midwifery at Guy’s Hospital. It was there that he began the experiments on transfusion that led to its rebirth. The frequency of postpartum hemorrhage and death troubled Blundell. In 1818 he wrote<sup>23</sup>:

A few months ago I was requested to visit a woman who was sinking under uterine hemorrhage . . . Her fate was decided, and notwithstanding every exertion of the medical attendants, she died in the course of two hours.

Reflecting afterwards on this melancholy scene . . . , I could not forbear considering, that the patient might very probably have been saved by transfusion; and that . . . the vessels might have been replenished by means of the syringe with facility and promptitude. This opening statement introduced Blundell’s epoch-making study titled “Experiments on the Transfusion of Blood by the Syringe.”<sup>23</sup> (See Fig 1-2.) Blundell described in detail a series of animal experiments. He demonstrated that a syringe could be

used effectively to perform transfusion, that the lethal effects of arterial exsanguination could be reversed by the transfusion of either venous or arterial blood, and that the injection of 5 drams (20 cc) of air into the veins of a small dog was not fatal but transfusion across species ultimately was lethal to the recipient.<sup>23</sup> Thus Blundell was the first to state clearly that only human blood should be used for human transfusion. The latter conclusion was confirmed in France by Dumas and Prevost, who demonstrated that the infusion of heterologous blood into an exsanguinated animal produced only temporary improvement and was followed by death within 6 days.<sup>24</sup> These scientific studies provided the basis for Blundell’s subsequent efforts in clinical transfusion.

The first well-documented transfusion with human blood took place on September 26, 1818.<sup>25</sup> The patient was an extremely emaciated man in his mid-thirties who had pyloric obstruction caused by carcinoma. He received 12 to 14 ounces of blood in the course of 30 or 40 minutes. Despite initial apparent improvement, the patient died 2 days later. Transfusion in the treatment of women with postpartum hemorrhage was more successful. In all, Blundell performed 10 transfusions, of which five were successful. Three of the unsuccessful transfusions were performed on moribund patients; the fourth was performed on a patient with puerperal sepsis; and the fifth was performed on the aforementioned patient with terminal carcinoma. Four of the successful transfusions were given for postpartum hemorrhage, and the fifth was administered to a boy who bled after amputation.<sup>22</sup> Blundell also devised various instruments for the performance of transfusion. They included an “impellor,” which collected blood in a warmed cup and “impelled” the blood into the recipient via an attached syringe, and a “gravitator”<sup>26</sup> (Fig 1-3), which received blood and delivered it by gravity through a long vertical cannula.

The writings of Blundell provided evidence against the use of animal blood in humans and established rational indications for transfusion. However, the gravitator (Fig 1-3) graphically demonstrated the technical problems that remained to be solved. Blood from the donor, typically the patient’s husband, flowed into a funnel-like device and down a flexible cannula into the patient’s vein “with as little exposure as possible to air, cold and inanimate surface.”<sup>25</sup> The amount of blood transfused was estimated from the amount spilled into the apparatus by the donor. In this clinical atmosphere, charged with apprehension and anxiety, the amount of blood issuing from a donor easily could be overstated. Clotting within the apparatus then ensured that only a portion of that blood actually reached the patient. Thus the amount of blood actually transfused may have been seriously overestimated. This may explain the apparent absence of transfusion reactions. Alternatively, reactions may have been unrecognized. Patients who underwent transfusion frequently were agonal. As Blundell<sup>26</sup> stated, “It seems right, as the operation now stands, to confine transfusion to the first class of cases only, namely, those in which there seems to be no hope for the patient, unless blood can be thrown into the veins.” Under these circumstances, “symptoms” associated with an “unsuccessful” transfusion might be ascribed to the agonal state rather than the

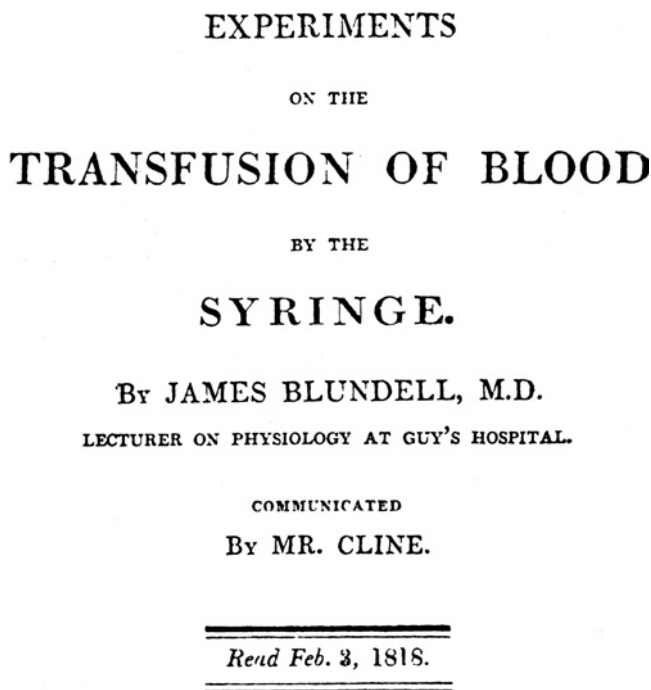


Figure 1-2. The beginnings of modern transfusion.<sup>23</sup>

transfusion itself. For a time, the problem of coagulation during transfusion was circumvented by the use of defibrinated blood. This undoubtedly increased the amount of blood actually transfused. However, there were numerous deaths. Interestingly, these deaths were attributed to intravascular coagulation when in actuality they were probably fatal hemolytic reactions caused by the infusion of incompatible blood.<sup>27</sup>

Transfusion at the end of the 19th century, therefore, was neither safe nor efficient. The following description, written in 1884, illustrates this point<sup>28</sup>:

Students, with smiling faces, are rapidly leaving the theatre of one of our metropolitan hospitals. The most brilliant operator of the day has just performed immediate transfusion with the greatest success. By means of a very beautiful instrument, the most complex and ingenious that modern science has yet produced, a skilful surgeon has transfused half a pint, or perhaps a pint, of blood from a healthy individual to a fellow creature profoundly collapsed from the effects of severe hemorrhage. Some little difficulty was experienced prior to the operation, as one of the many stop-cocks of the

transfusion apparatus was found to work stiffly; but this error was quickly rectified by a mechanic in attendance. Towards the close of the operation the blood-donor, a powerful and heavy young man, swooned. Two porters carried him on a stretcher into an adjoining room.

In the latter half of the 19th century, there were many attempts to render transfusion a more predictable and less arduous procedure. In 1869, Braxton-Hicks,<sup>29</sup> using blood anticoagulated with phosphate solutions, performed a number of transfusions on women with obstetric bleeding. Many of the patients were in extremis, and ultimately all died. Unfortunately, a detailed description of terminal symptoms was not provided.<sup>29</sup> Some investigators attempted to rejuvenate animal-to-human transfusion, and Oscar Hasse persisted in this approach despite disastrous results. Studies by Emil Ponfick and by Leonard Landois finally put an end to this practice. Ponfick, in carefully controlled studies, confirmed the lethality of heterologous transfusion and identified the resulting hemoglobinuria along with its donor erythrocyte source. Landois documented the poor results of animal-to-human transfusion and demonstrated the lysis of sheep erythrocytes by human serum in vitro.<sup>8</sup>

Frustration with blood as a transfusion product led to even more bizarre innovations. From 1873 to 1880, cow, goat, and even human milk was transfused as a blood substitute.<sup>30</sup> The rationale derived from an earlier suggestion that the fat particles of milk could be converted into blood cells. Milk transfusion was particularly popular in the United States,<sup>30</sup> where the practice of animal-to-human transfusion was recorded as late as 1890.<sup>31</sup> Fortunately, these astonishing practices were discontinued when saline solutions were introduced as "a life-saving measure" and "a substitute for the transfusion of blood."<sup>32</sup> A passage from an article written by Bull in 1884<sup>32</sup> is particularly instructive:

The danger from loss of blood, even to two-thirds of its whole volume, lies in the disturbed relationship between the calibre of the vessels and the quantity of the blood contained therein, and not in the diminished number of red blood-corpuscles; and ... This danger concerns the volume of the injected fluids also, it being a matter of indifference whether they be albuminous or containing blood corpuscles or not ...

Mercifully, volume replacement with saline solutions deflected attention from the unpredictable and still dangerous practice of blood transfusion. Accordingly, transfusions were abandoned until interest was rekindled by the scientific and technical advances of the early 20th century.

## The 20th Century

The 20th century was ushered in by a truly monumental discovery. In 1900, Karl Landsteiner (1868-1943) observed that the sera of some persons agglutinated the red blood cells of others. This study, published in 1901 in the *Wiener Klinische Wochenschrift*<sup>33</sup> (Fig 1-4), showed for the first time the cellular differences in

Vol. II.] LONDON, SATURDAY, JUNE 13. [1828-9.

### OBSERVATIONS ON TRANSFUSION OF BLOOD.

By DR. BLUNDELL.

*With a Description of his Gravitator.\**

STATES of the body really requiring the infusion of blood into the veins are probably rare; yet we sometimes meet with cases in which the patient must die unless such operation can be performed; and still more frequently with cases which seem to require a supply of blood, in order to prevent the ill health which usually arises from large losses of the vital fluid, even when they do not prove fatal.

\* The instrument is manufactured by Messrs. Maw, 35, Aldermanbury.

In the present state of our knowledge respecting the operation, although it has not been clearly shown to have proved fatal in any one instance, yet not to mention possible, though unknown risks, inflammation of the arm has certainly been produced by it on one or two occasions; and therefore it seems right, as the operation now stands, to confine transfusion to the first class of cases only, namely, those in which there seems to be no hope for the patient, unless blood can be thrown into the veins.

The object of the Gravitator is, to give help in this last extremity, by transmitting the blood in a regulated stream from one individual to another, with as little exposure as may be to air, cold, and inanimate surface; ordinary venesection being the only operation performed on the person who emits the blood; and the insertion of a small tube into the vein usually laid open in bleeding, being all the operation which it is necessary to execute on the person who receives it.

The following plate represents the whole apparatus connected for use and in action:—

Tab. 1.



No. 302.

Figure 1-3. Blundell's gravitator.<sup>26</sup>

**Aus dem pathologisch-anatomischen Institute in Wien.  
Ueber Agglutinationserscheinungen normalen  
menschlichen Blutes.**

Von Dr. Karl Landsteiner, Assistenten am pathologisch-anatomischen  
Institute.

**Figure 1-4.** Landsteiner's description of blood groups.<sup>33</sup>

individuals from the same species. In his article, Landsteiner wrote<sup>34</sup>:

In a number of cases (Group A) the serum reacts on the corpuscles of another group (B), but not, however, on those of group A, while, again, the corpuscles of A will be influenced likewise by serum B. The serum of the third group (C) agglutinates the corpuscles of A and B, while the corpuscles of C will not be influenced by the sera of A and B. The corpuscles are naturally apparently insensitive to the agglutinins which exist in the same serum.

With the identification of blood groups A, B, and C (subsequently renamed group O) by Landsteiner and of group AB by Decastello and Sturli,<sup>35</sup> the stage was set for the performance of safe transfusion. For this work, Landsteiner somewhat belatedly received the Nobel Prize in 1930. But even that high recognition does not adequately express the true magnitude of Landsteiner's discovery. His work was like a burst of light in a darkened room. He gave us our first glimpse of immunohematology and transplantation biology and provided the tools for important discoveries in genetics, anthropology, and forensic medicine. Viewed from this perspective, the identification of human blood groups is one of only a few scientific discoveries of the 20th century that changed all of our lives.<sup>34</sup> Yet the translation of Landsteiner's discovery into transfusion practice took many years.

At the turn of the century, the effective transfer of blood from one person to another remained a formidable task. Clotting, still uncontrolled, quickly occluded transfusion devices and frustrated most efforts. In 1901 the methods used in transfusion were too primitive to demonstrate the importance of Landsteiner's discovery. Indeed, the study of in-vitro red cell agglutination may have seemed rather remote from the technical problems that demanded attention. An intermediate step was needed before the importance of Landsteiner's breakthrough could be perceived and the appropriate changes could be incorporated into practice. This process was initiated by Alexis Carrel (1873-1944), another Nobel laureate, who developed a surgical procedure that allowed direct transfusion through an arteriovenous anastomosis.

Carrel<sup>36</sup> introduced the technique of end-to-end vascular anastomosis with triple-threaded suture material. This procedure brought the ends of vessels in close apposition and preserved luminal continuity, thus avoiding leakage or thrombosis. This technique paved the way for successful organ transplantation and brought Carrel the Nobel Prize in 1912. It was also adapted by Carrel<sup>37</sup> and others<sup>38,39</sup> to the performance of transfusion. Crile<sup>38</sup> introduced the use of a metal tube to facilitate placement of sutures, and Bernheim<sup>39</sup> used a two-piece cannula to unite the artery to the vein (Fig 1-5). Because all of these procedures usually culminated in the

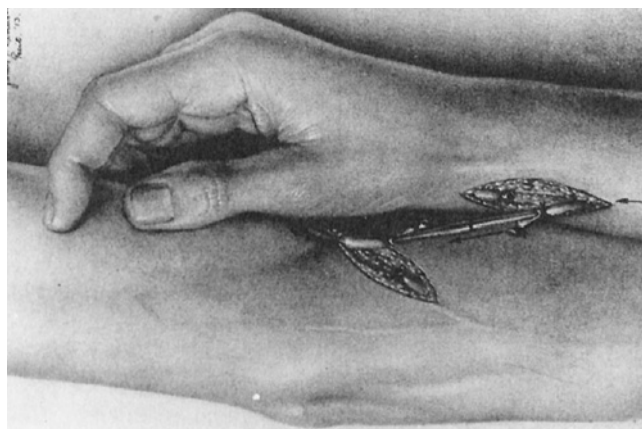


**Figure 1-5.** Direct transfusion by means of arteriovenous anastomosis through the two-pieced cannula of Bernheim.<sup>39</sup>

**A CASE OF FATAL HEMOLYSIS FOLLOWING  
DIRECT TRANSFUSION OF BLOOD BY  
ARTERIOVENOUS ANASTOMOSIS.\***

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AND

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PHILADELPHIA.

**Figure 1-6.** Report of a fatal transfusion reaction.<sup>41</sup>

sacrifice of the two vessels, they were not performed frequently. Direct transfusion was also fraught with danger. In a passage written two decades later, the procedure was recalled in the following manner<sup>40</sup>:

The direct artery to vein anastomosis was the best method available but was often very difficult or even unsuccessful. And, what was almost as bad, one never knew how much blood one had transfused at any moment or when to stop (unless the donor collapsed). (I remember one such collapse in which the donor almost died—and the surgeon needed to be revived.)

Despite these many difficulties, direct transfusion through an arteriovenous anastomosis for the first time efficiently transferred blood from one person to another. The process also disclosed fatal hemolytic reactions that were undeniably caused by transfusion<sup>41</sup> (Fig 1-6). However, the relation of these fatal reactions to Landsteiner's discovery was not recognized until Reuben Ottenberg (1882-1959) demonstrated the importance of compatibility testing.

Ottenberg's interest in transfusion began in 1906 while he was an intern at German (now Lenox Hill) Hospital in New York. There Ottenberg learned of Landsteiner's discovery and in 1907 began pretransfusion compatibility testing.<sup>42</sup> Ottenberg accepted an appointment at Mount Sinai Hospital the next year and continued



**ACCIDENTS IN TRANSFUSION**  
**THEIR PREVENTION BY PRELIMINARY BLOOD EXAMINATION:**  
**BASED ON AN EXPERIENCE OF ONE**  
**HUNDRED TWENTY-EIGHT TRANSFUSIONS \***

REUBEN OTTENBERG, M.D.

AND

DAVID J. KALISKI, M.D.

NEW YORK

Accidents following transfusion have been sufficiently frequent to make many medical men hesitate to advise transfusion, except in desperate cases. It has been our opinion since we began making observations on this question in 1908 that such accidents could be prevented by careful preliminary tests, leading to the exclusion of agglutinative or hemolytic donors. Our observations on over 125 cases have confirmed this view and we believe that untoward symptoms can be prevented with absolute certainty.

**Figure 1-7.** Report of the importance of testing before transfusion.<sup>43</sup>

his studies on transfusion. In 1913, Ottenberg published the report that conclusively demonstrated the importance of preliminary blood testing for the prevention of transfusion “accidents”<sup>43</sup> (Fig 1-7). This was not Ottenberg’s only contribution. He observed the mendelian inheritance of blood groups,<sup>44</sup> and he was the first to recognize the relative unimportance of donor antibodies and consequently the “universal” utility of type O blood donors.<sup>45</sup>

Further advances in immunohematology were to occur in succeeding decades. The M, N, and P systems were described in the period between 1927 and 1947.<sup>46</sup> The Rh system was discovered in connection with an unusual transfusion reaction. In 1939, Levine and Stetson<sup>47</sup> described an immediate reaction in a group O woman who had received her husband’s group O blood soon after delivery of a stillborn fetus with erythroblastosis. This sequence of events suggested that the infant had inherited a red cell agglutinin from the father that was foreign to the mother. At about the same time, Landsteiner and Wiener<sup>48</sup> harvested a rhesus monkey red cell antibody from immunized guinea pigs and rabbits. This antibody agglutinated 85% of human red cell samples (Rh-positive) and left 15% (Rh-negative) unaffected. When the experimentally induced antibody was tested in parallel with the serum from Levine’s patient, a similar positive and negative distribution was observed, and the Rh system had been discovered. Other red cell antigen systems were subsequently described, but when Rh Immune Globulin was introduced as a preventive measure for hemolytic disease of the newborn, it became one of the major public health advances of the century.

Despite the introduction of compatibility testing by Ottenberg, transfusion could not be performed frequently as long as arteriovenous anastomosis remained the procedure of choice. Using this method, Ottenberg needed 5 years (Fig 1-7) to accumulate the 128 transfusions he reported in his study on pretransfusion testing.<sup>43</sup> New techniques, such as Unger’s two-syringe method introduced in 1915<sup>49</sup> (Fig 1-8), eventually put an end to transfusion by means of arteriovenous anastomosis. However, transfusions did not become commonplace until anticoagulants were developed and direct methods of transfusion were rendered obsolete.

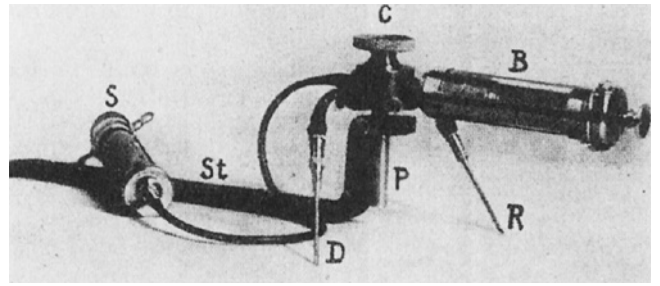


**Figure 1-8.** Apparatus for Unger’s two-syringe, four-way stopcock method of indirect transfusion.<sup>49</sup>

## Anticoagulants, the Blood Bank, and Component Therapy

The anticoagulant action of sodium citrate completely transformed the practice of transfusion. Early reports from Belgium<sup>50</sup> and Argentina<sup>51</sup> were followed by the work of Lewisohn<sup>52</sup> that established the optimal citrate concentration for anticoagulation. The work of Weil<sup>53</sup> then demonstrated the feasibility of refrigerated storage. Subsequently, Rous and Turner<sup>54</sup> developed the anticoagulant solution that was used during World War I.<sup>55</sup> Despite its very large volume, this solution remained the anticoagulant of choice until World War II, when Loutit and Mollison<sup>56</sup> developed an acid-citrate-dextrose (ACD) solution. Used in a ratio of 70 mL ACD to 450 mL blood, ACD provided 3 to 4 weeks of preservation of a more concentrated red cell infusion. Thus, the two world wars were the stimuli for the development of citrate anticoagulants and the introduction of indirect transfusion.<sup>46</sup> For the first time, the donation process could be separated, in time and place, from the actual transfusion. Blood drawn and set aside now awaited the emergence of systems of storage and distribution. Again, it was the provision of medical support during armed conflict that stimulated these developments.

A blood transfusion service, organized by the Republican Army during the Spanish Civil War (1936-1939), collected 9000 L of blood in citrate-dextrose anticoagulant for the treatment of battle casualties.<sup>57</sup> At about that same time, Fantus<sup>58</sup> began operation of the first hospital blood bank at Cook County Hospital in Chicago. His interest had been stimulated by Yudin’s report<sup>59</sup> on the use of cadaveric blood in Russia. Apart from certain scruples attached to the use of cadaveric blood, Fantus reasoned that a transfusion service based on such a limited source of supply would be impractical. Accordingly, he established the principle of a “blood bank” from which blood could be withdrawn, provided it had previously been deposited. As Fantus<sup>58</sup> himself stated, “just as one cannot draw money from a bank unless one has deposited some, so the blood preservation department cannot supply blood unless as much comes in as goes out. The term ‘blood bank’ is not a mere metaphor.” The development of anticoagulants and the concept of blood banks provided an infrastructure upon which a more elaborate blood services organization could be built. World War II was the catalyst for these further developments.

At the beginning of World War II, blood procurement programs were greatly expanded.<sup>46</sup> In Great Britain an efficient system had been developed through the organization of regional centers. When the war started, these centers, already in place, were able to increase their level of operation. In the United States the use of plasma in the management of shock had led to the development of plasma collection facilities.<sup>60</sup> The efficient long-term storage of plasma had been further facilitated by the process of lyophilization developed by Flosdorf and Mudd and the introduction of ABO-independent “universal” plasma produced by pooling of several thousand units of plasma.<sup>61</sup> In 1940, the United States organized a program for the collection of blood and the shipment of plasma to Europe. The American Red Cross, through its local chapters, participated in the project, which collected 13 million units by the end of the war.<sup>46</sup>

The national program of the American Red Cross ceased at the end of the war. However, many of the local chapters continued to help recruit donors for local blood banks, and in 1948, the first regional Red Cross blood center was begun in Rochester, New York. By 1949-1950 in the United States, the blood procurement system included 1500 hospital blood banks, 1100 of which performed all blood bank functions. There were 46 nonhospital blood banks and 31 Red Cross regional blood centers. By 1962, these numbers had grown to 4400 hospital blood banks, 123 nonhospital blood banks, and 55 American Red Cross regional blood centers, and the number of units collected had grown to between 5 and 6 million per year.<sup>62</sup>

During this time, blood was collected through steel needles and rubber tubing into rubber-stoppered bottles. After washing and resterilization, the materials were reused. On occasion, “vacuum bottles” were used to speed up the collection. However, the high incidence of pyrogenic reactions soon led to the development of disposable plastic blood collection equipment.

In a classic article written in 1952, Walter and Murphy<sup>63</sup> described a closed, gravity technique for whole blood preservation. They used a laminar flow phlebotomy needle, an interval donor tube, and a collapsible bag of polyvinyl resin designed so that the unit could be assembled and ready for use after sterilization with steam. The polyvinyl resin was chemically inert to biologic fluids and nonirritating to tissue. Soon thereafter, Gibson et al<sup>64</sup> demonstrated that plastic systems were more flexible and allowed removal of plasma after sedimentation or centrifugation. In time, glass was replaced with plastic, and component therapy began to emerge. This development was enhanced by the US military’s need to reduce the weight and breakage of blood bottles during shipment in the Korean War.

Component and derivative therapy began during World War II when Edwin J. Cohn and his collaborators developed the cold ethanol method of plasma fractionation.<sup>65</sup> As a result of their work, albumin, globulin, and fibrinogen became available for clinical use. As plastic equipment replaced glass, component separation became a more widespread practice, and the introduction of automated cell separators provided even greater capabilities in this area.

Clotting factor concentrates for the treatment of patients with hemophilia and other hemorrhagic disorders also were developed during the postwar era. Although antihemophilic globulin had been described in 1937,<sup>66</sup> unconcentrated plasma was the only therapeutic material until Pool discovered that Factor VIII could be harvested in the cryoprecipitable fraction of blood.<sup>67</sup> This resulted in the development of cryoprecipitate, which was introduced in 1965 for the management of hemophilia. Pool showed that cryoprecipitate could be made in a closed-bag system and urged its harvest from as many donations as possible. The development of cryoprecipitate and other concentrates was the dawn of a golden age in the care of patients with hemophilia. Self-infusion programs, made possible by technologic advances in plasma fractionation, allowed early therapy and greatly reduced disability and unemployment. This golden age came abruptly to an end with the appearance of the AIDS virus.

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## Transfusion in the Age of Technology

In contrast to the long ledger of lives lost in previous centuries because of the lack of blood, transfusion in the 20th century saved countless lives. In 1937, during those early halcyon days of transfusion, Ottenberg wrote<sup>40</sup>:

Today transfusion has become so safe and so easy to do that it is seldom omitted in any case in which it may be of benefit. Indeed the chief problem it presents is the finding of the large sums of money needed for the professional donors who now provide most of the blood.

It is ironic that Ottenberg’s statement should refer to paid donors and foreshadow difficulties yet to come. However, experience to that point had not revealed the problem of viral disease transmission. More transfusions would have to be administered before that problem would be perceived.

After the introduction of anticoagulants, blood transfusions were given in progressively increasing numbers. At Mount Sinai Hospital in New York, the number of blood transfusions administered between 1923 and 1953 increased 20-fold<sup>68,69</sup> (Table 1-1). This increase was particularly notable after the establishment of blood banks. It was during this period that Beeson wrote his classic description of transfusion-transmitted hepatitis<sup>70</sup> (Fig 1-9). He had been alerted to the problem by the outbreaks of jaundice that followed inoculation programs with human serum during World War II. Thus blood transfusion entered a new era. Blood components not only saved lives but also transmitted disease. The discovery of the Australian antigen<sup>71</sup> and the subsequent definition of hepatitis A virus and B virus (HBV) still left residual non-A and non-B disease,<sup>72</sup> a gap that has been largely filled by the discovery of the hepatitis C virus (HCV).<sup>73</sup> However, it was the outbreak of AIDS that galvanized public attention to blood transfusion.

The AIDS epidemic was first recognized in the United States, and the first case of AIDS associated with transfusion was observed in a 20-month-old infant.<sup>74</sup> Subsequently the suspicion that AIDS

**Table 1-1.** Increase in the Number of Blood Transfusions at Mount Sinai Hospital, New York, 1923-1953

| Year | No. of Transfusions  |
|------|----------------------|
| 1923 | 143                  |
| 1932 | 477                  |
| 1935 | 794                  |
| 1938 | (Blood bank started) |
| 1941 | 2097                 |
| 1952 | 2874                 |
| 1953 | 3179                 |

Adapted from Lewisohn.<sup>68</sup>**Table 1-2.** Transfusions in the United States (in Millions of Units)<sup>78-84</sup>

| Year | Whole Blood and Red Blood Cells | Platelets | Plasma | Total |
|------|---------------------------------|-----------|--------|-------|
| 1971 | 6.32                            | 0.41      | 0.18   | 6.91  |
| 1979 | 9.47                            | 2.22      | 1.29   | 12.98 |
| 1980 | 9.99                            | 3.19      | 1.54   | 14.72 |
| 1982 | 11.47                           | 4.18      | 1.95   | 17.60 |
| 1984 | 11.98                           | 5.53      | 2.26   | 19.77 |
| 1986 | 12.16                           | 6.30      | 2.18   | 20.64 |
| 1987 | 11.61                           | 6.38      | 2.06   | 20.05 |
| 1989 | 12.06                           | 7.26      | 2.16   | 21.48 |
| 1992 | 11.31                           | 8.33      | 2.26   | 21.90 |
| 1994 | 11.11                           | 7.87      | 2.62   | 21.60 |
| 1997 | 11.52                           | 9.04      | 3.32   | 23.88 |
| 1999 | 12.39                           | 9.05      | 3.32   | 24.76 |
| 2001 | 13.90                           | 10.20     | 3.93   | 28.03 |
| 2004 | 14.18                           | 9.88      | 4.09   | 28.15 |
| 2006 | 14.65                           | 10.39     | 4.01   | 29.05 |

## JAUNDICE OCCURRING ONE TO FOUR MONTHS AFTER TRANSFUSION OF BLOOD OR PLASMA

### REPORT OF SEVEN CASES

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The purpose of this communication is to report 7 cases of jaundice which occurred one to four months after transfusions of whole blood or plasma and to suggest that these illnesses were probably caused by the transfusions.

**Figure 1-9.** The first description of posttransfusion hepatitis. Used with permission from Beeson (JAMA).<sup>70</sup> Copyright 1943, American Medical Association.

could be transmitted by means of transfusion was confirmed.<sup>75</sup> The human immunodeficiency virus (HIV) was identified,<sup>76</sup> and an effective test to detect the HIV antibody was developed.<sup>76</sup>

## Concern for Blood Safety

Since 1943, transfusion therapy has been shadowed by the specter of disease transmission. In that year, Beeson described posttransfusion hepatitis and unveiled a problem that has grown with time. As transfusion increased, so did disease transmission. In 1962, the connection between paid donations and posttransfusion hepatitis was made.<sup>77</sup> A decade later, the National Blood Policy mandated a voluntary donation system in the United States. And yet, blood usage continued to increase.

Concern about posttransfusion hepatitis was not sufficient to decrease the number of transfusions. Although the use of whole blood declined as blood components became more popular, total blood use in the United States doubled between 1971 and 1980 (Table 1-2).<sup>78-84</sup> This pattern changed as the emergence of AIDS exposed all segments of society to a revealing light.

AIDS probably arose in Africa in the 1960s and spread quietly for years before it was detected. By 1980 an estimated 100,000 persons were infected, and by 1981, when the first cases were

reported, a worldwide pandemic lay just beneath the surface.<sup>85</sup> The initial response of the public and officials seemed trifling and insufficient as the outbreak grew to proportions few had foreseen. Criticism was levied against the news media for initially ignoring the story, the government for delay in acknowledging the problem, gay civil rights groups for resistance to epidemiologic measures, research scientists for unseemly competition, blood services for delayed response in a time of crisis, and the US Food and Drug Administration (FDA) for inadequate regulatory activity. Historians with the perspective of time will determine whether there really were more villains than the virus itself.<sup>86</sup>

Improved donor screening and increased donation testing have greatly decreased the risk of disease transmission and rendered the blood supply safer than it has ever been.<sup>86</sup> Nonetheless, the realization that transfusion can transmit an almost invariably fatal disease had a chilling effect on the public. Two major changes in blood services have occurred in the aftermath of the AIDS epidemic. The FDA, using pharmaceutical manufacturing criteria not "tailored to . . . blood banks," has become more aggressive in regulatory actions against blood collection establishments.<sup>87</sup> And, finally, blood use moderated for approximately 10 years. Through the 1980s and early 1990s, red cell and plasma transfusion peaked and began to stabilize (Table 1-2). Only platelet use and human progenitor cell transplantation, driven by the demands of cancer chemotherapy, continued to increase.<sup>79-81</sup> Educational programs to encourage judicious use of blood have been initiated, and they have been favorably received by practicing physicians.

Relentless public pressure for a "zero risk" blood supply resulted in dividends through continued scientific and technologic improvements. Enhanced sensitivity and better use of serologic testing, along with improved scrutiny of donors, resulted in major reductions in risk of transmitted disease by the mid-1990s.<sup>88</sup> Discovery that pools of units subjected to nucleic acid testing almost closed

the window for HIV and HCV virus resulted in application of this testing for both whole blood and plasma donations beginning between 1998 and 2000.<sup>89</sup> This, combined with virus reduction and inactivation of the final product, resulted in plasma derivatives that have not transmitted AIDS or hepatitis since 1994.<sup>90</sup> For whole blood and platelet components, risks have become low. A solvent/detergent-treated fresh frozen plasma component is used in Europe but not in the United States.

With the reduction in the risk of viral transmission, the focus in the developed world has shifted to transfusion-related acute lung injury—possibly from recipient-directed leukocyte antibodies and lipid mediators in transfused plasma—and bacterial infection primarily occurring in room-temperature stored platelets. So that incremental gains can be made against these risks, the use of male plasma only and the culture of platelets before they are released are being discussed and considered in some areas and implemented in others. Geographic exclusions have been aimed at reducing the potential for variant Creutzfeldt-Jakob disease (vCJD) transmission by transfusion, although in the United States such occurrence seems highly unlikely. In many countries, universal leukocyte reduction has been a response to the vCJD risk. Ironically universal application of leukocyte reduction is probably ineffective for vCJD but has stimulated a controversy in the United States over its use for preventing other problems.<sup>91</sup>

Finally, focus on the understanding, management, and prevention of medical errors in general might lead to progress against remaining nemesis hemolytic transfusion reactions caused by mistransfusion. Bar code technology at the bedside, commonly applied to prevent errors in medication administration, has shown efficacy in reducing transfusion errors.<sup>92</sup> Radio-frequency identification shows further promise in error-prone situations such as operating rooms.<sup>93</sup> Transfusion safety officers and hemovigilance systems are other initiatives being considered or instituted.

“Zero risk” has still not been achieved. Emerging global infections such as West Nile virus, Chagas’ disease, and Chikungunya virus remain future potential threats and have encouraged further test development. Nevertheless, increased public and physician confidence in the safety of the blood supply (Table 1-3) combined with both increased aggressiveness of therapies and aging of the population resulted in increased blood use by 1999. For 2001 through 2006, total red cell transfusions in the United States increased by 5.4% and total platelet and plasma transfusions rose by 2% each. A significant decrease was seen in autologous transfusions (47.4%).<sup>84</sup>

Current Megatrends

In the developed world, no cost has been spared in meeting public demands for blood safety. Service fees charged to hospitals by independent blood centers in the United States (Fig 1-10) illustrate the effect each new safety development has had on the cost of Red Blood Cells (RBCs). As the new millennium began, the

Table 1-3. Risk Estimates per Unit of Red Blood Cells Transfused in the United States, Ranked by Frequency

| Type of Risk                       | Estimated Occurrence    |
|------------------------------------|-------------------------|
| Urticaria                          | 1 in 50-100             |
| Red cell allommunization           | 1 in 100                |
| Febrile reaction                   | 1 in 300                |
| TRALI                              | 1 in 5000               |
| Hemolytic reaction                 | 1 in 6000               |
| Transfusion to the wrong recipient | 1 in 14,000-19,000      |
| Anaphylaxis                        | 1 in 20,000-50,000      |
| Hepatitis B virus                  | 1 in 100,000-200,000    |
| HTLV-I/II                          | 1 in 641,000            |
| Hepatitis C virus                  | 1 in 1-2 million        |
| HIV                                | 1 in 2-3 million        |
| Malaria                            | 1 in 4 million          |
| Bacterial contamination            | 1 in 5 million          |
| GVHD                               | Very rare, no estimates |

TRALI = transfusion-related acute lung injury; HTLV-I/II = human T-cell lymphotropic virus, types I and II; HIV = human immunodeficiency virus; GVHD=graft-vs–host disease Adapted from Klein et al.<sup>94</sup>

mean payment for RBC units was \$100, and a leukocyte-reduced unit was \$126. By 2005 those had risen to \$157 and \$188 respectively, with significant annual increases.<sup>95</sup>

One group of researchers (committed to programs to reduce blood use) has published data suggesting that the societal cost of a unit of RBCs is \$1400 per unit taking into account not only the blood center fee but also hospital-related costs, costs of treating adverse reactions, litigation, lost productivity of donors, and hemovigilance.<sup>96</sup> Although the figure might be overinflated, such work does highlight the ever increasing cost of this form of therapy to the patient and society.

In the underdeveloped world, the picture is quite different. The greatest blood need is for women hemorrhaging during childbirth, infants and children with anemia caused by malaria, and victims of trauma. In 80 of 172 countries responding to a World Health Organization (WHO) survey, fewer than 1% of the population donate blood. In sub-Saharan Africa, fewer than 3 million units of blood are collected annually for a population of more than 700 million. Of the 148 countries reporting data to WHO, 41 are unable to screen for minimum safety (HIV, HBV, HVC, and syphilis). WHO estimates that unsafe blood in these countries results in 16 million new infections with HBV, 5 million with HCV, and 160,000 with HIV each year (accounting for 5%-10% of the world’s HIV infections). Fortunately there is progress in some nations in achieving an all-volunteer supply and minimum screening. Thus, there are two drastically different pictures of blood safety and availability worldwide.<sup>97</sup>

Even in the developed world, availability remains a challenge. In the United States, the number in the population eligible to donate blood with all the new restrictions is 111 million, rather than the 177 million previously thought.<sup>98</sup> Finding ways to motivate sufficient numbers of people to donate remains

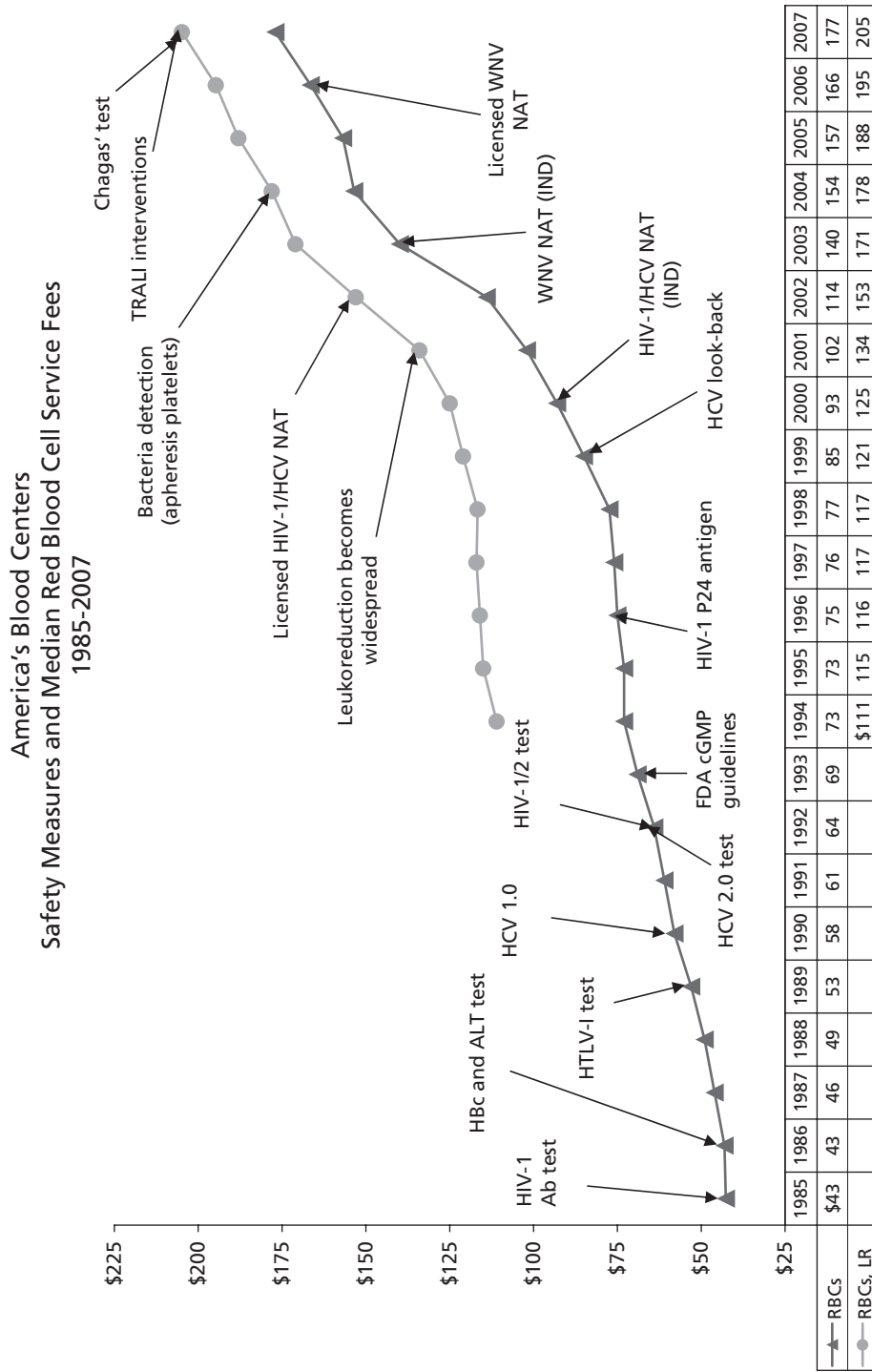


**Figure 1-10.** Red Blood Cell service fees charged by community blood centers from 1985 to 2007, correlated with safety measures. Courtesy of America's Blood Centers (modified). RBCs = Red Blood Cells; LR = RBCs Leukocytes Reduced; HIV = human immunodeficiency virus (-1/2 = types 1 and 2); Ab = antibody; HBc = hepatitis B core (antigen); ALT = alanine aminotransferase; HTLV-I = human T-cell lymphotropic virus, type I; HCV = hepatitis C virus; FDA cGMP = Food and Drug Administration current good manufacturing practice; NAT = nucleic acid testing; IND = investigational new drug; WNV = West Nile virus.

difficult. One suggestion has been to “personalize” the benefits. An example of this approach is having donors meet face-to-face with groups of recipients. Another response has been the use of cognitive interview evaluation and focus groups to define a more user-friendly questionnaire with computer-assisted techniques in many centers.<sup>99</sup> More aggressive marketing to growing minority populations as well as continued use of various incentives seem to have increased donations in some locations. A clear need is for more group O red cells; populations of non-European ethnicity generally have an increased proportion of group O.<sup>100</sup>

Another approach to maintain adequate availability is to control usage by ensuring that blood is used appropriately. Some US blood centers have been successful in bringing their transfusion medicine expertise into the patient-care setting by providing transfusion services to hospitals. The model in Seattle, WA, has operated for decades.<sup>101,102</sup> In the United Kingdom, liaison systems for blood centers to hospitals employing Web-based technology for supply chain management have been introduced.<sup>103,104</sup> In Denmark, success has been reported using the Thromboelastograph (Haemoscope, Niles, IL) hemostatic system to manage coagulopathy in conjunction with treating physicians—something also done in many US hospitals.<sup>105</sup> Other point-of-care tests to assess the state of the coagulation system and tissue oxygenation could also result in more accurately targeted component transfusion. Transfusion medicine specialists in hospitals—whether from pathology groups, blood center staff, or other areas such as anesthesiology or hematology—are critical to the successful use of blood transfusion in patient care.

Although some advances in transfusion medicine at the end of the last millennium have been difficult to implement, such as use of hemoglobin solutions and some pathogen inactivation technologies, the field has continued to advance into new areas of stem cell biology, regenerative medicine, and cord blood banking. In addition, transfusion medicine specialists increasingly function in collaboration with surgeons, oncologists, and hematologists in treating the acutely ill patient with complex medical problems. With all the added sophistication, the optimal hemoglobin and platelet triggers and endpoints for transfusion remain unsettled. Clinicians are less likely to use oxygenation transport endpoints to determine the need for red cell transfusion but are beginning to look for other means to assess tissue oxygenation. If a patient’s hemoglobin is too high (even when below normal), complications such as thromboembolism can result. Too low an endpoint exposes some patients to the risk of tissue hypoxia. The clearest trend has been away from autologous transfusion, although some medical centers seek bloodless medicine and surgery combining pharmacotherapy (mainly erythropoietin and iron), blood recovery and reinfusion, and conservative triggers and endpoints.<sup>94</sup> More conservative triggers and endpoints for platelet transfusion are becoming accepted, but approaches to alloimmunized patients and bacterial contamination are still in question.<sup>106</sup> Also debatable is whether transfusion, through some poorly quantifiable mechanism such as immunomodulation, confers a poorer prognosis on acutely ill patients.

From ancient times into the new millennium, blood has been a substance that fascinates mankind. Despite unresolved

controversies, blood transfusion remains of critical importance in the care of sick patients throughout the world.

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## Disclaimer

The authors have disclosed no conflicts of interest.

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# I Blood Components and Derivatives

## 2

## Red Cell Production and Kinetics

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The main function of erythrocytes (red cells) is to transport oxygen from the lungs to the other tissues of the body. This delivery of oxygen is finely controlled by the number of circulating erythrocytes, which is a function of the rate of old erythrocyte removal from the circulation and the rate of new erythrocyte production. Circulating erythrocytes are maintained in an extremely narrow range because the normal marrow produces almost exactly the same number of new erythrocytes each day as is lost through senescence. This daily turnover of about 1% of circulating erythrocytes represents approximately 250 billion erythrocytes in a healthy adult. When much greater numbers of circulating erythrocytes are lost, such as with bleeding or hemolysis, the production of new erythrocytes increases rapidly to maintain the steady-state number of erythrocytes. This rapid expansion of erythrocyte production in response to bleeding or hemolysis is so well regulated that rebound polycythemia or overproduction of erythrocytes does not occur. This exquisitely controlled production of erythrocytes is mediated through a negative feedback mechanism that involves oxygen delivery to, and utilization by, the kidneys, the hormone erythropoietin (EPO) that is produced by specialized cells in the kidneys, and the erythroid progenitor cells in the marrow that respond to EPO. Normal red cell production also depends upon adequate supplies of specific nutrients, among which folate, vitamin B<sub>12</sub>, and iron are the most important in terms of clinically encountered anemias. Disorders of the hematopoietic system or other disease processes such as chronic inflammation also affect the erythropoietic process.

**Erythropoiesis****Erythropoiesis: A Component of Hematopoiesis**

*Erythropoiesis*, the process of erythrocyte production, is a component of the larger process of hematopoiesis in which a stem

cell proliferates and differentiates to form all the cell types in the blood and immune system.<sup>1</sup> The endproducts of hematopoiesis include platelets, granulocytes, monocytes-macrophages, T lymphocytes, and B lymphocytes as well as erythrocytes. Thus, normally regulated hematopoiesis is required for effective hemostasis, inflammation, immune response, and tissue oxygenation. Among these various functions, tissue oxygenation is the first required during embryonic development and the most tightly regulated in postnatal life. Erythropoiesis has two sequential phases during development—the primitive phase, during weeks 3 to 8 of human gestation, when erythrocytes are produced in “blood islands” of the yolk sac, and the subsequent definitive phase in which erythrocytes are produced in the fetal liver and spleen and later in the marrow.<sup>2-4</sup> In humans, the hemoglobin of the primitive erythrocytes contains embryonic  $\epsilon$ - and  $\zeta$ -globins, whereas the hemoglobin of the definitive erythrocytes contains adult  $\alpha$ -globin and either fetal  $\gamma$ -globin from midgestation through the first few postnatal months or adult  $\beta$ -globin after the first few postnatal months. Embryologic studies demonstrate that the primitive and definitive phases of erythropoiesis arise from different stem cells in the early embryo.<sup>2,3,5,6</sup> The primitive erythroid cells arise in the inner cell mass of the yolk sac blood islands, whereas the definitive erythroid cells arise from the aortogonadomesonephros region of the mesoderm.

Current concepts of hematopoiesis are derived mainly from studies of mice and humans. These studies have included direct morphologic and immunologic analyses of cells in hematopoietic tissues, in-vitro culture of hematopoietic progenitor cells and hematopoietic stromal cells, transplantation studies with hematopoietic cells, and genetic studies of mice with natural mutations, transgene expressions, or targeted gene knockouts. Hematopoietic stem cells (HSCs) appear to be immediate progeny of an even more primitive stem cell, the hemangioblast, which can give rise to both hematopoietic cells and vascular cells during embryonic development.<sup>7</sup> Studies with HSCs have led to a hematopoietic model (Fig 2-1) that applies to postnatal as well as prenatal hematopoiesis because turnover of both blood and immune cells requires new cell production throughout life. In this model, pluripotent HSCs, which have an incidence in

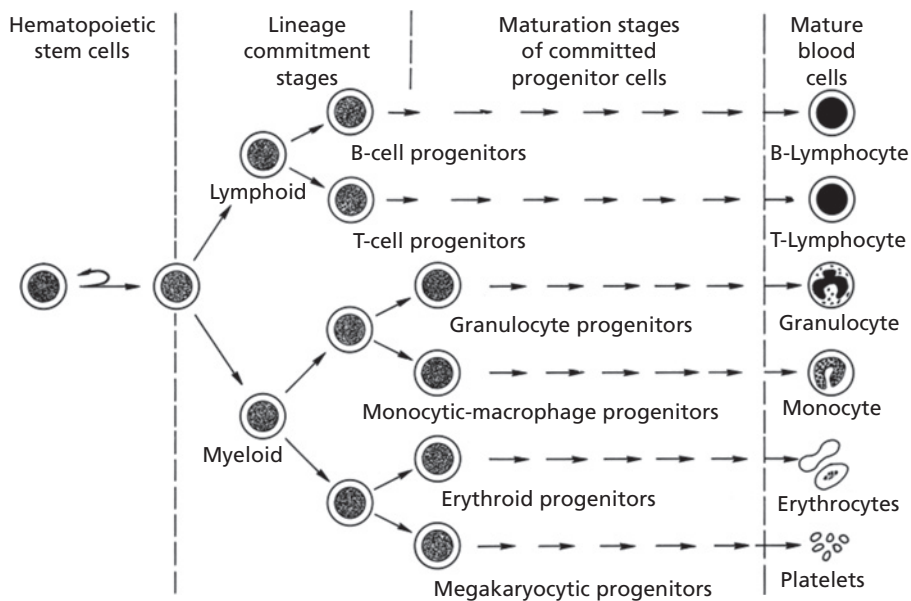


**Figure 2-1.** Hematopoietic stem cell differentiation. Pluripotent hematopoietic stem cells (HSCs) can self-replicate (curved arrow) or give rise to progenitor cells committed to differentiation. Because of uncertainty about events leading to commitment and about self-replication of HSCs around the time of commitment, a hypothetical cell is shown at the border between the HSC and lineage commitment stages. Progeny of HSCs become committed to differentiation along specific cell lineages. After lineage commitment, progenitor cells mature and acquire the characteristics of terminally differentiated cells in blood (eg, erythroid cells produce hemoglobin and enucleate). During this maturation phase, lineage-specific hematopoietic growth factors act on progenitor cells. The stages of HSC differentiation are shown, but the proliferation that accompanies the differentiation is not. Proliferative potential decreases as cells progress through various stages toward mature blood cells. (Used with permission from Koury et al.<sup>8</sup>)

marrow of one per  $10^4$  to  $10^5$  nucleated cells, have two functions—self-replication and production of progenitor cells committed to differentiation into all lymphoid and myeloid cell types. Differentiation of HSCs requires a process called *commitment* in which the stem cell loses self-renewal capacity and is limited to either further differentiation or death. Commitment restricts the hematopoietic progenitor cells to differentiation along either the lymphoid or the myeloid pathway and subsequently to the specific, single-cell lineages (Fig 2-1). Differentiation involves cellular proliferation and additional commitment steps.

Two possible mechanisms of hematopoietic cell commitment are a deterministic one and a stochastic one.<sup>1</sup> In the deterministic mechanism, molecules from the cell's environment react with specific receptors on or in the cell and thereby lead to changes in specific gene structure or expression that, in turn, determine the commitment status of that cell. These environmental molecules can be either soluble growth factors or fixed ligands on other cells or extracellular matrix components in the hematopoietic microenvironment. An example of deterministic commitment is Indian hedgehog upregulating the expression of bone morphogenic protein-4 (BMP-4) in the developing mesodermal cells.<sup>9</sup> BMP-4, in turn, induces hematopoietic and endothelial differentiation, with the ventral portion of the developing dorsal aorta and the subjacent mesoderm inducing definitive hematopoiesis.<sup>9</sup> In the stochastic mechanism, the fate of a hematopoietic cell is determined by the outcome of random intracellular events that

change the structure or expression of specific genes.<sup>10,11</sup> Two examples of such changes in hematopoietic cells are immunoglobulin gene rearrangement in B-lymphocyte progenitors and binding of specific transcription factors such as TAL-1 or GATA-1 to the promoters of erythroid-specific genes.<sup>12,13</sup> With the stochastic mechanism, environmental signals such as the concentration of a specific hematopoietic growth factor, or the interactions with marrow stromal cells, promote survival or proliferation but do not affect commitment. Thus, these environmental factors influence the size of hematopoietic cell populations that have had commitment decisions made by random intracellular events.

### Stages of Erythropoiesis

The stages of erythropoiesis from HSCs and extending through the mature erythrocytes are shown in Fig 2-2. Hematopoietic cell transplantation experiments with mice have demonstrated murine marrow cells that possess the properties of HSCs.<sup>14-16</sup> Through analyses of murine HSCs and comparisons with human hematopoietic cell populations, a subset of hematopoietic cells that is highly enriched in human HSCs can be identified with cellular markers.<sup>1</sup> This human HSC-enriched population displays the CD34 and CD90 (Thy 1) antigens and has low uptake of rhodamine-123 and weak expression of HLA-DR antigens.

After erythroid lineage commitment, the first stage of recognized differentiation is the *burst-forming units–erythroid* (BFU-E)

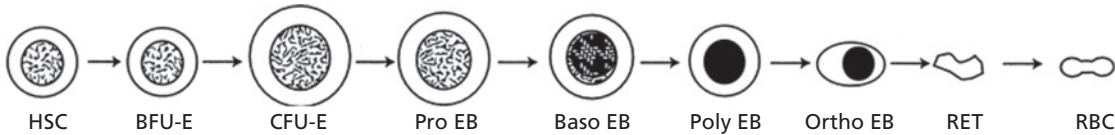
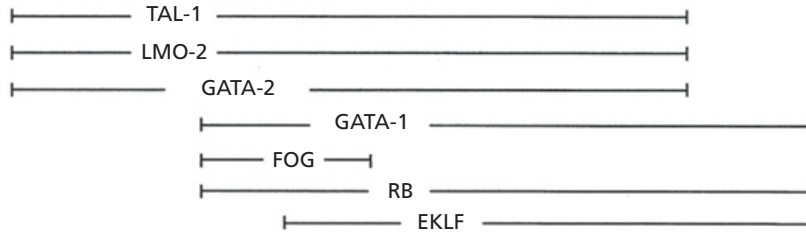
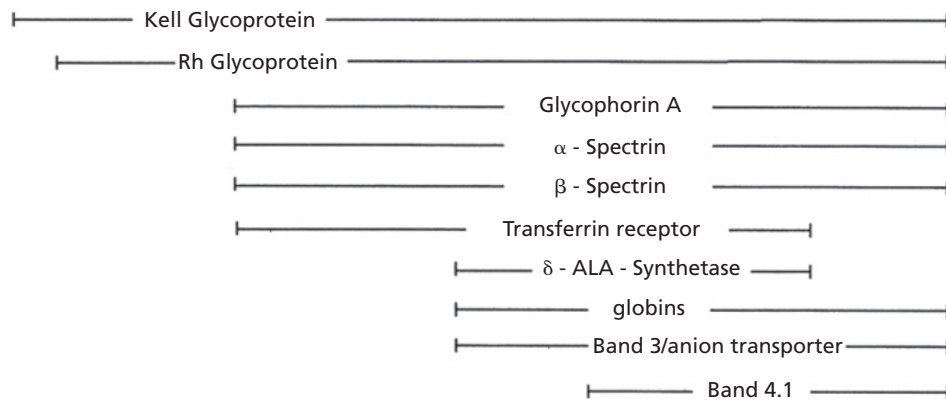
**(A) Stage of erythroid differentiation****(B) Transcription factors for erythroid differentiation****(C) Receptors for required hematopoietic growth factors****(D) Proteins related to erythrocyte structure and function**

**Figure 2-2.** Cellular events in erythroid differentiation. (A) The relative sizes and the known or presumed morphologic appearances of erythroid cells at various stages of differentiation—pluripotent hematopoietic stem cells (HSCs); burst-forming unit–erythroid (BFU-E); colony-forming unit–erythroid (CFU-E); proerythroblasts (Pro EB); basophilic erythroblasts (Baso EB); polychromatophilic erythroblasts (Poly EB); orthochromatic erythroblasts (Ortho EB), reticulocytes (RET); erythrocytes (RBCs). (B) Erythroid transcription factors—basic helix-loop-helix factor (TAL-1); Lim-domain partner of TAL-1 (LMO-2); zinc finger factors that bind GATA sequences (GATA-1, GATA-2); GATA-1 partner, “friend of GATA” (FOG); retinoblastoma protein (RB); erythroid Krüppel-like factor (EKLF). (C) Receptors for hematopoietic growth factors—stem cell factor receptor (c-KIT); erythropoietin receptor (EPO-R); insulin-like growth factor-1 receptor (IGF-1-R). (D) Proteins related to erythrocyte structure and function. Periods of expression for erythroid-specific forms of proteins are shown. Transferrin receptors are present in all stages, but a period of large up-regulation is shown. Rh-associated glycoprotein (Rh50) is expressed before the proerythroblast stage, while the RhD and RhCE proteins are first expressed in the proerythroblast stage or later. For each transcription factor, growth factor receptor, and erythrocyte-related protein, the degree of expression can vary greatly during the period shown.

that proliferates and differentiates in semisolid tissue culture medium to form large colonies or groups of colonies called *bursts of erythroblasts*.<sup>17</sup> Human BFU-Es need 2 to 3 weeks of culture to form bursts that contain as many as several thousand erythroblasts. The more immature BFU-Es need the longest culture period and form the largest bursts. The next well-defined stage of erythroid development is the *colony-forming unit–erythroid* (CFU-E).<sup>18</sup> Human CFU-Es need 7 days in vitro to proliferate and differentiate into small colonies, usually composed of up to 64 erythroblasts. After the CFU-E stage is the proerythroblast

stage, the last erythroid progenitor with a nonspecific morphologic appearance in the differentiation scheme shown in Fig 2-2. The proerythroblast gives rise to the successive stages of the basophilic, polychromatophilic, and orthochromatic erythroblasts that are recognized by light microscopy of stained marrow samples. Orthochromatic erythroblasts enucleate, forming reticulocytes. Reticulocytes are very irregularly shaped cells containing residual organelles (the “reticulum”) that allow the cells to be distinguished from the more mature erythrocytes. The final stage of differentiation, the erythrocyte, is achieved after the reticulocytes

rid themselves of their residual organelles and remodel their irregular shapes to become uniform biconcave disks. Each progenitor cell loses proliferative potential as it differentiates along this continuum until it reaches the late erythroblast stages in which the cells do not divide. Thus, between the BFU-E and the CFU-E stages are a series of intermediate stages called *mature burst-forming units*, which need more than 1 week but less than 2 weeks in culture to develop into colonies with sizes intermediate between those of typical erythroid bursts and colonies.<sup>19</sup> Similarly, the reticulocyte population has early, intermediate, and late stages of development based on their relative RNA content, which steadily declines with maturation.<sup>20</sup>

### Requirements for Normal Erythroid Differentiation

A series of intracellular and extracellular events are needed for successful completion of the erythroid differentiation scheme as shown in Fig 2-2. The intracellular events include the expression of 1) general hematopoietic and erythroid-specific transcription factors, 2) receptors for hematopoietic growth factors, and 3) proteins such as hemoglobin, membrane, and membrane cytoskeleton proteins found in the mature erythrocyte. Synthesis of hemoglobin begins as the erythroblasts are making the transition from the basophilic to the polychromatophilic stages. Among the erythroid membrane proteins, the Kell and the Rh glycoproteins are expressed before the CFU-E stage, while most of the other membrane proteins found on mature erythrocytes are expressed at the proerythroblast stage or later.<sup>21,22</sup> At the proerythroblast stage,  $\alpha$ - and  $\beta$ -spectrin are synthesized, but they are degraded in the cytoplasm rather than associating with the cytoskeleton.<sup>23</sup> Glycophorin A is present in the plasma membrane of proerythroblasts,<sup>21,22</sup> but the band 3/anion transporter protein is not made until the basophilic erythroblast stage, and band 4.1 does not begin to accumulate until the polychromatophilic stage.<sup>23</sup> Once the band 3/anion transporter protein is present in the membrane,  $\alpha$ - and  $\beta$ -spectrin become membrane associated in the characteristic 1:1 ratio.<sup>23</sup>

Extracellular requirements for erythroid differentiation include 1) the adequate supply of required hematopoietic growth factors, 2) a sufficient supply of nutrients required for progenitor cell proliferation and differentiation, and 3) the presence of stromal cell and matrix support. In Fig 2-2, the transcription factors, growth factor receptors, and hematopoietic growth factors necessary for normal erythropoiesis are shown for the period of differentiation when they are known to be needed. The principal growth factor that regulates erythropoiesis is EPO, which is discussed in detail in the following section. The nutrients that play important roles in erythropoiesis—folate, vitamin B<sub>12</sub>, and iron—are required at all stages of erythroid cell differentiation, but most importantly in the later stages. Deficiency of any of these three nutrients results in decreased erythrocyte production and anemia. The roles of these nutrients in erythropoiesis are described in later sections. HSCs and BFU-Es are found in the hematopoietic organs, but they also can circulate in the blood. Erythroid cells in the CFU-E through orthochromatic stages of

differentiation do not circulate and, in the hematopoietic tissue, they form specific structures with stromal macrophages termed erythroblastic islands.<sup>24</sup> An erythroblastic island consists of a central macrophage surrounded by the adherent erythroid cells that are usually at about the same stage of differentiation within each individual island. Ablation of the stromal macrophages impairs the erythropoietic response to blood loss, indicating that erythroblastic islands have a functional role in red cell production.<sup>25</sup> At least five surface membrane proteins can mediate erythroid-macrophage interactions in erythroblastic islands.

1. Vascular cell adhesion molecule 1 (VCAM-1) on marrow macrophages binds  $\alpha 4 \beta 1$  integrin (also known as very late antigen-4 or VLA-4) on erythroblasts.<sup>26</sup>
2.  $\alpha_V$  component of integrins (eg,  $\alpha_V \beta 1$ ,  $\alpha_V \beta 3$ , and  $\alpha_V \beta 5$ ) on marrow macrophages binds interstitial cell adhesion molecule 4 (ICAM-4, also known as LW glycoprotein) on erythroblasts.<sup>27</sup>
3. Erythroblast-macrophage protein (EMP), a transmembrane protein of both erythroblasts and macrophages, mediates interactions between these two cell types through a homophilic reaction.<sup>28</sup>
4. Sialoadhesin (CD169 or Siglec 1) on macrophages binds sialylated glycoproteins on erythroblasts.<sup>29</sup>
5. Hemoglobin-haptoglobin receptor (CD163) is a marrow macrophage surface glycoprotein that binds an unknown ligand on erythroblasts.<sup>30</sup>

Each of these adhesion protein pairs can be disrupted by antibodies and/or competing peptide ligands such that binding of erythroblasts to macrophages is inhibited. Because disruption of one of these binding protein pairs can inhibit attachment of erythroblasts to macrophages, the proteins may form functional binding complexes on the surfaces of macrophages and erythroblasts.

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## Erythropoietin

### Regulation of Erythropoietin Production by Tissue Hypoxia

Under normal circumstances, the rate of erythrocyte production is controlled by the interaction of EPO with the EPO receptors on the erythroid progenitor cells. Erythropoietin is a 30.4-kD glycosylated protein, most of which is produced by the kidneys.<sup>31</sup> The kidneys are a major component of the oxygenation-EPO negative feedback mechanism shown in Fig 2-3. The other components are the marrow, the blood erythrocytes, and EPO itself. The negative feedback characteristics account for the fine control of erythrocyte numbers that occur in each individual. The major determinant of oxygen delivery from the lungs to the peripheral tissues is the number of circulating erythrocytes. When erythrocyte numbers decrease, as occurs with bleeding or hemolysis, oxygen delivery decreases and the peripheral tissues become hypoxic. All tissues experience hypoxia, but the major ones that respond with EPO production are the kidneys and the liver.

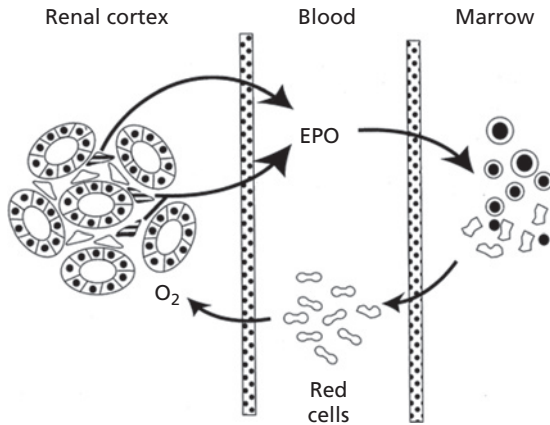


**Figure 2-3.** The oxygenation-erythropoietin (EPO) negative feedback mechanism. The number of circulating red cells determines the amount of oxygen delivered from the lungs to other tissues. In the renal cortex, a specific subset of interstitial cells produce EPO when they perceive hypoxia. The EPO is immediately secreted into the blood and acts in the marrow to prevent the programmed death (apoptosis) of erythroid progenitor cells. Those erythroid progenitors that survive the EPO-dependent period of differentiation mature into reticulocytes (irregularly shaped anucleate cells in marrow and blood) and subsequently into erythrocytes. Increased numbers of erythrocytes resulting from increased plasma EPO levels deliver more oxygen to the kidneys and thereby lower the amount of EPO produced as renal hypoxia is relieved.

The same cells that sense hypoxia are the ones that produce EPO.<sup>32</sup> The mechanism for sensing hypoxia in cells involves a family of specific transcription factors, hypoxia-inducible factors (HIFs).<sup>33,34</sup> HIFs have two components, HIF- $\alpha$  and HIF- $\beta$ , which form a complex that binds to a hypoxia-inducible transcription enhancer located in the region of various genes that include *EPO*, vascular endothelial growth factor (*VEGF*), and genes encoding several of the glycolytic enzymes.<sup>35</sup> In the case of *EPO*, HIF-2 $\alpha$  is the family member that binds to a 120-bp enhancer 3' to the human *EPO* polyadenylation signal<sup>36,37</sup> as shown in Fig 2-4(B). HIF-1 $\beta$  is constitutively produced, and its intracellular levels are not influenced by hypoxia. HIF- $\alpha$  components are constitutively produced, but under normoxic conditions, they are essentially undetectable because they are rapidly degraded by the ubiquitin-proteasome pathway,<sup>38</sup> shown in Fig 2-4(A). However, when an EPO-producing cell experiences hypoxia, this rapid degradation ceases and intracellular HIF-2 $\alpha$  levels promptly increase, as in Fig 2-4(B). Polyubiquitination of HIF-2 $\alpha$  depends upon the von Hippel-Lindau protein (pVHL) interacting with those HIF-2 $\alpha$  molecules that have hydroxylation of two specific proline residues.<sup>39–41</sup> Hydroxylation of a specific asparagine residue<sup>42,43</sup> in HIF-2 $\alpha$  prevents its interaction with the p300 member of the HIF transcription complex, thereby preventing the formation of the active complex. These hydroxylations are directly linked to the oxygenation of the EPO-producing cell because the two non-heme iron-containing hydroxylases, which catalyze these proline and asparagine hydroxylation reactions respectively, use molecular oxygen as a substrate.<sup>35</sup> Under

normoxic conditions, the hydroxylations and subsequent ubiquitination and interference with transcription complex formation proceed, but with hypoxia they do not. These posttranslational regulations of HIF-2 $\alpha$  levels and activity are the key components in controlling *EPO* gene transcription. Components of this complex other than HIF-2 $\alpha$  and HIF-1 $\beta$  include hepatocyte nuclear factor-4 (HNF-4) and the protein p300.<sup>44–46</sup> Once hypoxia reaches the threshold that triggers *EPO* transcription, the resultant *EPO* messenger RNA is translated into the EPO glycoprotein, which is immediately secreted.<sup>31</sup> When an individual cell is triggered to produce EPO, it does so in an all-or-nothing manner.<sup>32,47</sup> Thus, EPO concentrations in the blood increase sharply within 2 hours after loss of blood, hemolysis, or a sudden decrease in atmospheric oxygen.

The kidney cells that produce EPO are a subset of cortical interstitial cells adjacent to proximal tubules<sup>48,49</sup> and appear to be fibroblasts<sup>50,51</sup> (Fig 2-3). In mild anemia, small foci of the EPO-producing cells are present in the inner cortex. In moderate anemia, the EPO-producing cells are present in larger areas within the inner half of the cortex. In severe anemia, they are present throughout the renal cortex.<sup>47</sup> These progressive increases in the areas of EPO production in the kidney correspond to increasing areas of renal cortical hypoxia that are a function of oxygen supply from the blood and local oxygen utilization by rapidly metabolizing cells, such as the adjacent proximal tubular epithelium.<sup>47,52</sup> Thus, the rapid increase in EPO production after blood loss or hemolysis is not due to increased production by each EPO-producing cell but rather to the recruitment to active EPO production of increased numbers of cells with the potential to produce EPO.<sup>47</sup> The number of cells actively producing EPO and the resultant plasma EPO levels increase exponentially with a linear decrease in hematocrit. This exponential increase characterizes most anemias except for those involving patients with renal disease or malignancies.<sup>53</sup>

### Interaction of Erythropoietin and Erythroid Progenitor Cells

EPO is carried through the blood to the marrow, where it binds to the specific transmembrane glycoprotein erythropoietin receptor (EPO-R).<sup>54</sup> The mature, fully glycosylated EPO-R is displayed on the surface of erythroid progenitor cells at the CFU-E stage and persists through the late basophilic erythroblast stage with an average of approximately 1000 surface receptors per cell<sup>55,56</sup> (Fig 2-2). The EPO-R is structurally homologous with receptors for numerous hematopoietic growth factors and cytokines including granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, thrombopoietin, the interleukins, growth hormone, and prolactin.<sup>57</sup> The binding of EPO to EPO-Rs leads to three major events: 1) homodimerization and conformational alterations of EPO-Rs, 2) initiation of intracellular signaling by the EPO-Rs, and 3) endocytosis of the EPO/EPO-R complexes, which are subsequently proteolyzed.<sup>45,55,58–61</sup> Dimerization and structural changes of EPO-Rs after EPO binding induce both signaling and

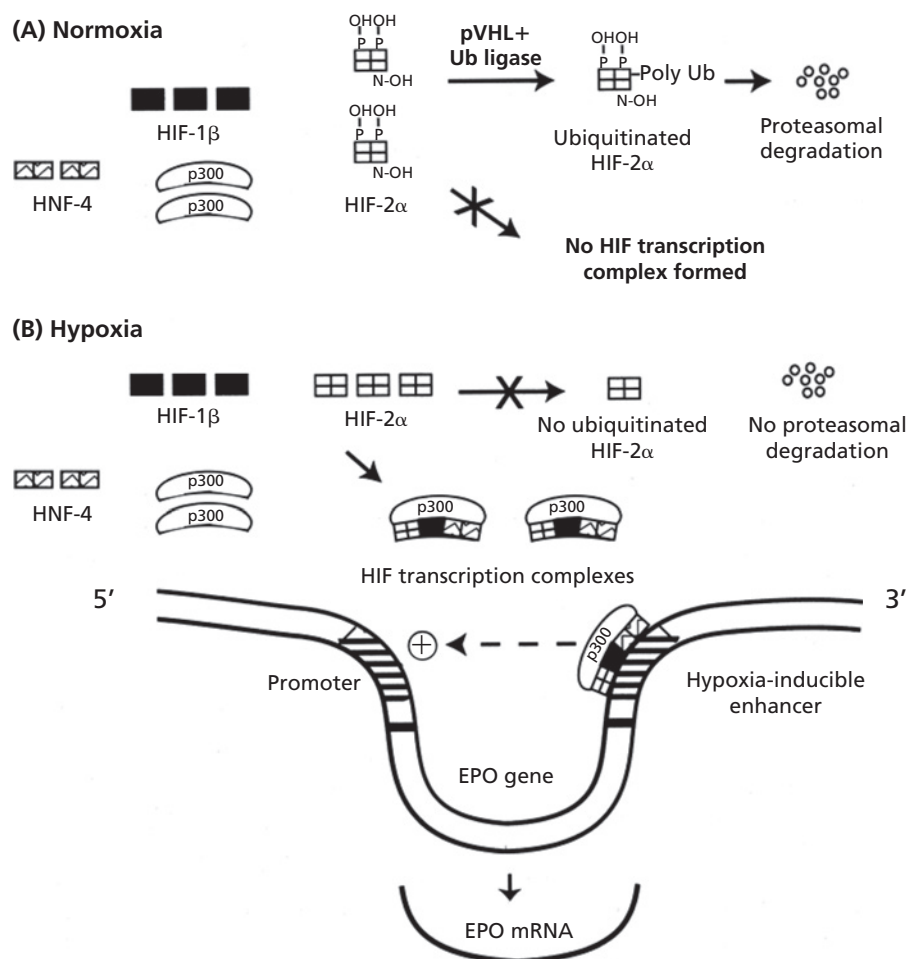


**Figure 2-4.** Induction of erythropoietin (EPO) gene transcription by hypoxia. (A) In cells capable of producing EPO, two components of hypoxia-inducible factor (HIF-2 $\alpha$  and HIF-1 $\beta$ ) are constitutively produced under normoxic conditions. However, the molecular oxygen present in the EPO-producing cells under normoxic conditions is used to hydroxylate two prolines and one asparagine in HIF-2 $\alpha$ . The prolyl hydroxylations (P-OH) lead to recognition by von Hippel-Lindau protein (pVHL), which targets HIF-2 $\alpha$  for polyubiquitination (Poly Ub) by ubiquitin ligase. The ubiquitinated HIF-2 $\alpha$  is rapidly degraded in proteasomes. Asparaginyl hydroxylation (N-OH) inhibits the association of the HIF-2 $\alpha$  with the p300 member of the HIF transcription complex, providing another means of blocking

the transcription of *EPO*. (B) When EPO-producing cells are hypoxic, HIF-2 $\alpha$  is not hydroxylated and accumulates because it is not degraded by the ubiquitin-proteasomal pathway. The HIF-2 $\alpha$  forms heterodimers with HIF-1 $\beta$ . Because the asparagine hydroxylation of HIF-2 $\alpha$  has not occurred when the EPO-producing cells are hypoxic, these heterodimers associate with two other components of the HIF transcription complex, hepatocyte nuclear factor-4 (HNF-4) and p300. The HIF transcription complex binds to the hypoxia-inducible enhancer 120 bp 3' to the *EPO* polyadenylation signal and increases *EPO* promoter activity with a resultant increase in *EPO* transcription and accumulation of EPO messenger RNA accumulation.

endocytosis. The endocytosis and intracellular degradation of the EPO/EPO-R complexes appear to be the normal mechanisms for clearance of EPO from the blood.<sup>62,63</sup>

Like the other hematopoietic growth factor-cytokine receptors, EPO-Rs have no intrinsic enzyme activity,<sup>57</sup> but they interact with several signal transduction pathways through Janus tyrosine kinase-2 (JAK2). JAK2 is physically associated with the cytoplasmic portion of EPO-Rs, where it chaperones EPO-Rs to the surface of the erythroid cell and is the initial enzyme activated by the conformational changes in the EPO-Rs produced by the binding of EPO.<sup>64,65</sup> JAK2 is activated by phosphorylation. In turn, activated JAK2 phosphorylates tyrosines in EPO-Rs and initiates signal transduction pathways that include signal

transduction and activator of transcription-5 (STAT5), RAS-raf-MAP kinase, and phosphoinositol-3 kinase/Akt kinase (protein kinase B).<sup>45,55,58-61</sup> In addition to activating these pathways that appear to lead to increased erythroid cell numbers, JAK2 also activates SHP-1 and SHP-2 phosphatases and suppressors of cytokine signaling-3 (SCOS-3) that control the phosphorylation state of the EPO-Rs and ultimately cease EPO-R signaling.<sup>66-68</sup>

### Effects of Erythropoietin on Erythroid Progenitor Cells

EPO has been proposed to be an inducer of proliferation and terminal differentiation, but the only effect of EPO on erythroid progenitor cells that has been well documented is the prevention of programmed death (apoptosis). Promotion of survival



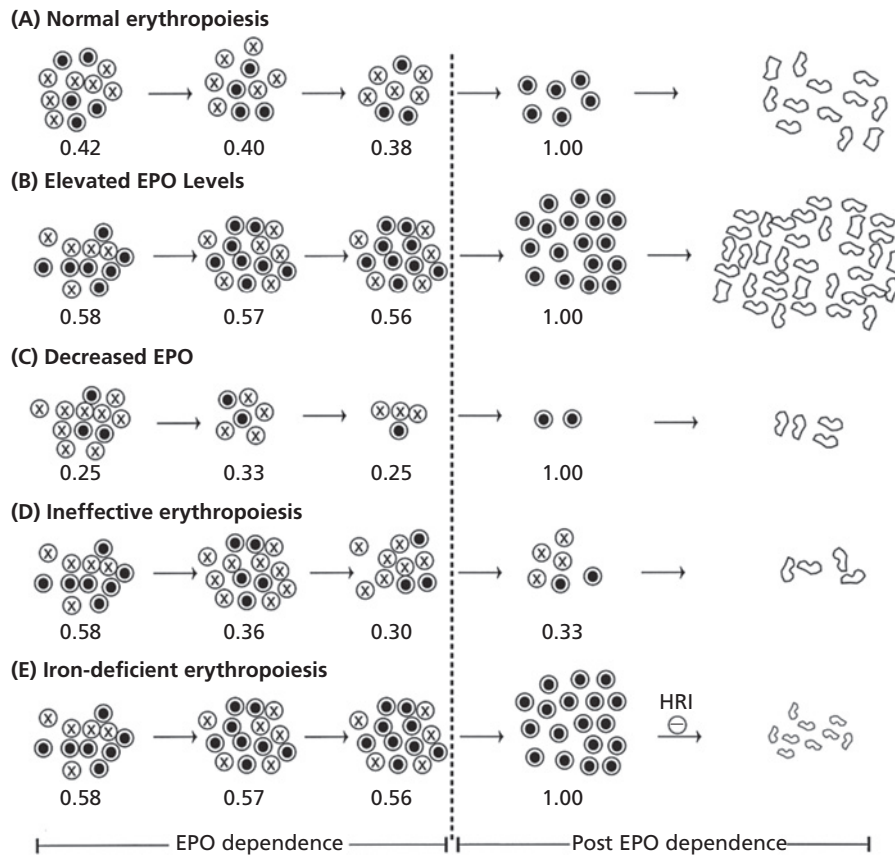


**Figure 2-5.** Model of erythropoiesis based on suppression of programmed cell death (apoptosis) by erythropoietin (EPO) and heterogeneity in EPO dependence among erythroid cells. Between the (A) Normal erythropoiesis with an average survival rate of 40% in each of the EPO-dependent generations. Normal erythropoiesis produces about 250 billion new erythrocytes daily, despite the fact that a minority of all potential erythroid cells survive the EPO-dependent period. (B) Elevated EPO levels as found after acute blood loss or hemolysis increase average survival rates to 57% in each EPO-dependent generation. Daily erythrocyte production is increased three times the normal amount. (C) Decreased EPO levels as found in renal failure decrease average survival rate to 28% in each EPO-dependent generation. Daily erythrocyte production is one-third of normal.

(D) Ineffective erythropoiesis with high EPO levels increases rates of apoptosis caused by a pathologic process such as folate or vitamin B<sub>12</sub> deficiency. High EPO levels in response to decreased erythrocyte production expand surviving cells in the early EPO-dependent generations, but the increased rates of apoptosis in the late EPO-dependent and post-EPO-dependent stages decrease daily erythrocyte production to one-third of normal. (E) Iron-deficient erythropoiesis with elevated EPO levels resulting in a similar increase to an average of 57% survival as seen in (B), but in the post-EPO-dependent period, when hemoglobin is synthesized, heme-regulated inhibitor (HRI) prevents apoptosis by inhibiting protein synthesis. The inhibited protein synthesis decreases the size of erythrocytes produced and reduces daily erythrocyte production to three-fourths of the normal numbers.

by EPO through the prevention of apoptosis has been found in several different systems, including CFU-Es from various hematopoietic tissues<sup>69-72</sup> and in knockout mice that are either *EPO* null or *EPO-R* null.<sup>73,74</sup> These studies all indicate that erythroid progenitor cells in CFU-E through basophilic erythroblast stages are dependent upon EPO for their survival. How EPO signaling prevents apoptosis is unknown, but one key modulator of apoptosis, the anti-apoptotic protein Bcl-X<sub>L</sub>, has been implicated in erythroid cell survival.<sup>75,76</sup> Bcl-X<sub>L</sub>, however, does not mediate the anti-apoptotic effect of EPO on CFU-Es and proerythroblasts, but prevents the apoptosis of later stage erythroblasts.<sup>77</sup>

During the period of EPO dependence, individual erythroid cells in the same stage of differentiation from the same hematopoietic tissue display wide variation in degree of dependence on EPO for survival.<sup>78</sup> Some of the erythroid cells need very low levels of EPO,

such as those in the plasma of patients with chronic renal failure. Other erythroid cells need very high levels of EPO, such as those in patients with acute blood loss or hemolysis. Thus, this broad spectrum of EPO requirements covers the more than 1000-fold range of EPO concentrations found in plasma under various physiologic and pathologic conditions. The mechanism responsible for this heterogeneity in EPO dependence is not known, but it does not appear to be caused by differences in either the numbers of EPO-Rs or the EPO-binding affinities of EPO-Rs in the dependent progenitors.<sup>78</sup>

A model that incorporates the wide range of plasma EPO levels and the wide heterogeneity in EPO dependence among the EPO-responsive cells of the hematopoietic tissues has been proposed to explain the regulation of erythrocyte production by EPO in various physiologic and pathologic conditions.<sup>79</sup> In a version of this model in Fig 2-5, erythroid progenitor cells enter



an EPO-dependent period of differentiation (left of the dotted line in the figure) that extends from the CFU-E through the early erythroblast stages and encompassing three generations of cells. In Fig 2-5, the proportion of total cells that survive in a generation is shown immediately under the population. The surviving cells are represented by circles, each of which contains a large black dot representing an intact nucleus. The cells lost to apoptosis are shown by circles, each of which contains an X. The number of surviving cells in a generation results in twice that number for the total cells in the subsequent generation. Most cells reaching the CFU-E stage need more EPO than the low levels found in normal plasma to sustain them through the EPO-dependent period of differentiation. As a result, the approximately 250 billion erythrocytes produced daily by a normal, healthy adult are the progeny of a minority of all the erythroid progenitor cells that reach the CFU-E stage, shown in Fig 2-5(A).

When blood loss, hemolysis, or decreased atmospheric oxygen is encountered, plasma EPO increases, allowing the survival of many of EPO-dependent progenitors that would die by apoptosis under normal conditions. This enhanced survival increases reticulocyte production within a few days after encountering blood loss or decreased atmospheric oxygen. The increased reticulocytosis leads to increasing erythrocyte numbers until oxygen delivery recovers to normal, and then plasma EPO levels decline toward normal. In pathologic states of decreased oxygen delivery, such as lung disease or cardiac diseases with right-to-left shunts, the persistently increased EPO levels allow greater-than-normal survival of EPO-dependent cells such that the total number of erythrocytes is maintained in the polycythemic range. Conversely, when plasma EPO levels fall below normal because of decreased production in chronic renal disease, many erythroid progenitor cells that would survive the EPO-dependent period of differentiation under normal conditions die by apoptosis, resulting in anemia from decreased reticulocyte production, shown in Fig 2-5(C). Treatment of patients with renal failure with exogenously administered EPO rescues the erythroid progenitor cells that need normal plasma levels of EPO to survive.

## Nutritional Requirements for Erythropoiesis

Although erythropoiesis is finely regulated by the oxygenation-EPO feedback mechanism, the erythropoietic process is frequently limited by an insufficient supply of three essential nutrients. These nutrients are folate, vitamin B<sub>12</sub>, and iron. Although all proliferating cell populations need folate and vitamin B<sub>12</sub>, the large number of erythrocytes that must be produced each day results in a large DNA synthesis requirement for erythropoiesis. Although iron also is needed by all proliferating cell populations, the erythroblasts need much more iron than any other cell type because they produce hemoglobin. When any of these three nutrients is not present in sufficient amounts for the erythroid cell population of the marrow, production of erythrocytes decreases, and anemia occurs. Through the hypoxia

feedback mechanism, these anemias increase EPO production,<sup>53</sup> but increased EPO can only partially compensate for the decreased erythropoiesis caused by the specific nutrient deficiency. Administration of the deficient nutrient, however, results in resolution of anemia in each of the deficiency states.

Folate deficiency results in decreased intracellular levels of all folate coenzymes in the erythroid progenitor cells. Deficiency of vitamin B<sub>12</sub> results in trapping of folate in the methyltetrahydrofolate form, making it unavailable in the methylenetetrahydrofolate form required for thymidine synthesis or the formyltetrahydrofolate form required for purine synthesis.<sup>80</sup> As a result, vitamin B<sub>12</sub> deficiency, like folate deficiency, leads to decreased intracellular levels of the specific folate coenzymes needed for de novo synthesis of all of the deoxynucleotides used in DNA synthesis, except for deoxycytidine.<sup>81</sup> The inability to synthesize DNA caused by an inadequate supply of thymidine and purines causes accumulation of erythroid progenitors in the S phase of the cell cycle, which is rapidly followed by the induction of apoptosis.<sup>82,83</sup> The stages of erythroid differentiation that appear to be most susceptible to this apoptosis are at the end of the EPO-dependent stage and the beginning of the period of hemoglobin synthesis. EPO-induced expansion of the EPO-dependent population at the CFU-E and proerythroblast stages leads to the presence of even greater numbers of these progenitor cells that subsequently undergo apoptosis just as they are beginning to produce hemoglobin.<sup>84</sup> The resultant clinical disease is megaloblastic anemia. The prominent feature of megaloblastic anemia is ineffective erythropoiesis, shown in Fig 2-5(D). In ineffective erythropoiesis, progenitor cells in the EPO-dependent period expand in response to increased EPO levels. The number of reticulocytes formed, however, is less than normal because of the increased rates of pathologic apoptosis in the EPO-dependent and post-EPO-dependent periods of differentiation. The increased death of erythroid cells in ineffective erythropoiesis is recognized clinically by increases in iron turnover, serum bilirubin, and serum lactate dehydrogenase.

Erythroid progenitor cells are the greatest consumers of iron in the body. Iron uptake is mediated through transferrin receptors and tightly regulated by a complex intracellular system such that erythroid progenitor cells synthesize large amounts of hemoglobin without having an excess of iron, heme, or globins in the cells.<sup>85</sup> Two-thirds of the body's iron is in the hemoglobin of circulating erythrocytes, and iron deficiency almost always arises from blood loss. Two milliliters of blood contain 1 mg of iron, which is the amount normally absorbed each day from the diet. The iron absorption rate can be increased only slightly, so that chronic blood loss of just a few milliliters per day leads to iron deficiency. With iron deficiency anemia, serum EPO levels are elevated<sup>53</sup> and the number of CFU-Es in the hematopoietic organs are increased while the numbers of erythroblasts in the marrow and the circulating reticulocytes are decreased.<sup>86</sup> However, unlike megaloblastic anemia, ineffective erythropoiesis does not occur, and serum bilirubin and lactate dehydrogenase levels remain normal. The iron-deficient erythroid cells are

spared from apoptosis by one of the regulators of protein synthesis, heme-regulated inhibitor (HRI).<sup>87</sup> HRI is a protein kinase that phosphorylates eukaryotic translation initiation factor-2 $\alpha$  (eIF-2 $\alpha$ ), thereby inhibiting translation of globin (and other proteins) in erythroblasts. When free heme is present in the erythroid cells, it binds HRI and inhibits its phosphorylation of eIF-2 $\alpha$ , thereby permitting active globin (and other) protein translation. Conversely, when erythroblasts are iron-deficient, they produce much less heme and HRI phosphorylation of eIF-2 $\alpha$  is uninhibited, leading to greatly decreased rates of protein synthesis. This decreased protein synthesis results in a much slower rate of erythrocyte production and microcytosis.<sup>87</sup> The effect of iron deficiency and HRI on erythropoiesis is shown in Fig 2-5(E).

### **Influence of Pathologic States on Erythropoiesis**

The erythropoietic process can be influenced by diseases that are intrinsic to the erythropoietic cells or that secondarily affect the erythropoietic cells. Among the intrinsic diseases are several that decrease the number of erythropoietic cells. These include myelodysplasia, myeloid leukemia, aplastic anemia, pure red cell aplasia, and Fanconi anemia. Increased apoptosis of hematopoietic cells or failure to differentiate, in the case of acute leukemia, are considered the mechanisms of population reduction in these diseases. Conversely, polycythemia vera results in increased numbers of erythropoietic progenitor cells that have an increased sensitivity to EPO.<sup>88</sup> This increased sensitivity to EPO has been found to be caused by mutations in the JAK2 kinase that is responsible for the signaling of the EPO-R.<sup>89</sup> Indeed, the large majority of patients with polycythemia vera have a specific mutation in JAK2 kinase that keeps it in a constitutively active state. Because JAK2 also functions in the signaling of other hematopoietic growth factors, other hematopoietic lineages are involved in polycythemia vera as well as in other myeloproliferative disorders such as essential thrombocythemia and idiopathic myelofibrosis.<sup>89</sup> In patients with polycythemia vera the EPO feedback mechanism shown in Fig 2-3 is functional as demonstrated by lower-than-normal EPO levels, but the erythroid progenitor cells in the EPO-dependent stages in polycythemia vera survive much better with the low EPO levels than do normal erythroid progenitors with normal EPO levels.

Diseases that can secondarily decrease erythropoiesis include those that directly displace the hematopoietic cells in the marrow, such as metastatic neoplasms, lymphoid neoplasms, and myelofibrosis. However, the most common cause of secondary inhibition of erythropoiesis is anemia of chronic disease. This anemia occurs in chronic infections, neoplasms, and inflammatory diseases, and the prominent factor in its pathophysiology is excessive iron sequestration by macrophages. The major source of iron for erythropoiesis is macrophages, which store and process absorbed iron and recycled iron from the breakdown of

senescent erythrocytes.<sup>86</sup> Macrophages export iron to plasma transferrin, which delivers it to the erythroblasts. The release of iron from the macrophages occurs through ferroportin, a membrane transport protein. Ferroportin also mediates the passage of iron from duodenal enterocytes into the plasma where it is carried by transferrin. The expression of ferroportin is regulated by hepcidin, a 25-amino acid hormone produced in the liver.<sup>90</sup> Hepcidin production is induced by increased plasma iron and by cytokines produced in inflammation.<sup>90</sup> Hepcidin binds to ferroportin on the plasma membranes of macrophages and duodenal enterocytes, thereby inducing the internalization and degradation of the ferroportin.<sup>90</sup> Thus, increased hepcidin decreases ferroportin expression, resulting in the inhibition of iron export into the plasma by the macrophages and enterocytes. Therefore, as a result of hepcidin induction, less iron is available to erythroblasts and the erythropoietic process is suppressed.

Although iron sequestration by macrophages plays a major role, other direct and indirect effects of inflammatory cytokines on the erythropoietic cells have been reported in the anemia of chronic disease. Another indirect effect is decreased EPO production by the kidneys (relative to the degree of anemia), while direct effects on erythroid cells include suppressed growth and increased apoptosis induced by inflammatory cytokines such as interferon- $\gamma$ , tissue necrosis factor- $\alpha$ , and interleukin-1.<sup>91,92</sup> Although the anemia of chronic disease may partially respond to high doses of exogenously administered EPO, resolution of the anemia requires effective treatment of the primary disease responsible for increased cytokine production.

### **Summary**

Erythropoiesis is a component of the larger process of hematopoiesis, in which a pluripotent HSC gives rise through proliferation and differentiation to all the mature cells of the blood and the immune system. Within the erythroid differentiation process, the rate of erythrocyte production is regulated by EPO. EPO acts by increasing the number of erythroid progenitors that can survive during a specific period when they are dependent on EPO for the prevention of apoptosis. Levels of EPO are controlled by oxygen delivery and utilization in the renal cortex, where hypoxia induces EPO production. This oxygen-EPO feedback mechanism results in finely controlled rates of erythrocyte production. This mechanism responds promptly to physiologic changes such as blood loss or changes in atmospheric oxygen. In pathologic conditions, it provides compensatory changes in the rates of erythrocyte production that partially correct the abnormal oxygen delivery to the peripheral tissues. Although the use of EPO in clinical medicine is routine for patients with the anemia of renal disease, further research is needed to understand the intracellular signaling by the EPO-R that leads to increased erythroid cell survival in disorders such as myelodysplasia and the anemia of chronic disease. Understanding of these EPO-related mechanisms as well as the roles of HRI in preventing apoptosis of

iron-deficient erythroblasts and hepcidin in inducing the anemia of chronic disease will facilitate treatment of these various anemias.

## Disclaimer

The author has disclosed no conflicts of interest.

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# 3

## Regulation of Oxygen Delivery by Red Cells and Red Cell Substitutes

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The maintenance of tissue levels of  $O_2$  adequate to support aerobic respiration depends on four processes. The mechanics of breathing draw  $O_2$  into the alveolar space and expel  $CO_2$ . These respiratory gases are exchanged between the alveoli and the red cells in the pulmonary capillaries followed by transport of the oxygen-rich red cells to the peripheral circulation. In the last process,  $O_2$  diffuses along a concentration gradient from the red cells, through the blood vessel wall, into the tissue, and eventually to the mitochondria, where its reduction along the respiratory chain drives the generation of energy in the form of adenosine triphosphate (ATP). The body stores of  $O_2$  are small relative to the rate of consumption and cannot be drawn down too far before weakening the concentration gradients that are key determinants of tissue oxygenation. Disruption of any one of these processes impairs cellular oxygenation; hence, they are tightly regulated by multiple, overlapping systems that operate on the systemic, regional, and tissue levels. This chapter focuses on the regulation of the last phase of this process, the transport of oxygenated red cells to the periphery and delivery of oxygen to tissues.

### Regulation of Systemic Oxygen Delivery

Some of the quantitative aspects of systemic oxygen dynamics are reviewed in Chapter 9, which points out that the amount of oxygen delivered is a function of the unit volume of blood flow (related to cardiac output) and the oxygen content of blood, which is determined by the concentration of hemoglobin and the degree to which it is saturated with  $O_2$ . Ordinarily, the amount of oxygen delivered to the periphery exceeds what is required to sustain metabolic activity. Under resting conditions, only about 25% of all delivered  $O_2$  is consumed; hence, blood returning to the heart remains approximately 75% saturated. Even if delivery decreases because of reduced cardiac output or hemoglobin concentration,

or consumption increases because of exercise or fever, more oxygen can be extracted from the blood. However, at some threshold the tissues can no longer extract more oxygen and consumption becomes supply limited. To avoid reaching this point, systemic oxygen delivery is protected by regulation of the cardiac output, as well as the level and function of erythrocytic hemoglobin.<sup>1</sup> In normovolemic anemia, decreased blood viscosity contributes to facilitated venous return with increased preload and decreased afterload.<sup>2</sup> Anemia also results in increased synthesis of 2,3-diphosphoglycerate (2,3-DPG) in the red cell, which increases the hemoglobin  $P_{50}$  and facilitates oxygen release. In addition, blood flow is redistributed to tissues with increased metabolic needs (eg, heart and muscle in exercise) and diverted from tissues whose activity can be reduced or whose baseline oxygen extraction ratio is low (eg, splanchnic beds). Overlapping regulatory systems sensitive to blood volume, pressure, and oxygen levels trigger hemodynamic and metabolic responses that serve to maintain blood flow and oxygen delivery to the periphery. (See Table 3-1.)

### Central Nervous System Regulation of Hemodynamics

The central nervous system (CNS) regulates hemodynamic responses to hypovolemia and hypotension by increasing cardiac output and vascular tone and shunting blood flow away from such tissues as the skin and gut. Cardiac output is increased because of increases in both heart rate and stroke volume in the awake patient<sup>3</sup> but stroke volume alone in the anesthetized patient.<sup>4</sup> This regulatory response is initiated in part by vascular mechanoreceptors sensitive to stretch. High-pressure baroreceptors are located in the carotid bodies and the aortic arch, and low-pressure receptors are found in the heart and lungs.<sup>5,6</sup> The carotid sinus baroreceptors are located in the adventitia of the internal carotid artery, close to its juncture with the common carotid artery. Stretching of the arterial wall distorts sensory nerve endings and activates ion channels, causing membrane depolarization and the generation of an action potential proportionate to the degree of stretch. In the aortic arch, this signal transduction appears to be mediated by KCNQ  $K^+$  channels,<sup>7</sup> a group of widely distributed, multimeric ion channels that play a major role in determining neuronal excitability as well as in the

**Table 3-1.** Systemic Control of Blood Flow

| Type of Control | Input                                                 | Effector Organ                      | Output                                     |
|-----------------|-------------------------------------------------------|-------------------------------------|--------------------------------------------|
| Nervous         | High-pressure baroreceptors—aortic arch, carotid body | Medulla                             | Sympathetic/parasympathetic                |
|                 | Low-pressure baroreceptors—heart, lungs               | Medulla                             | Sympathetic/parasympathetic                |
|                 | Oxygen sensors—carotid body, medulla                  | Medulla                             | Sympathetic/parasympathetic                |
| Hormonal        | Baroreceptors                                         | Heart                               | BNP—vasodilation, natriuresis, diuresis    |
|                 | Baroreceptors—luminal [NaCl]                          | Juxtaglomerular cells of the kidney | Renin→→aldosterone—antidiuretic            |
|                 | Baroreceptors—aortic arch, carotid body               | Posterior pituitary gland           | Vasopressin—vasoconstriction, antidiuretic |
|                 | Osmoreceptors—hypothalamus                            | Posterior pituitary gland           | Vasopressin—vasoconstriction, antidiuretic |

BNP = B-type natriuretic peptide.

epileptic and arrhythmia syndromes that result from their malfunction.<sup>8</sup> Afferent impulses from the carotid mechanoreceptors are transmitted via sensory fibers of the carotid sensory nerve that travel with the glossopharyngeal nerve and project to the nucleus of the solitary tract in the medulla.<sup>5</sup> This nucleus also receives signals from the mechanoreceptors in the aortic arch and heart via nodose and vagal afferents, respectively. The baroreceptive neurons of the solitary tract nucleus in turn activate parasympathetic neurons of the dorsal vagal nucleus and the nucleus ambiguus. They also inhibit the sympathoexcitatory neurons of the rostral ventrolateral nucleus of the medulla.<sup>5,9</sup> Thus, activation of the mechanoreceptors by increased stretch (increased blood volume or pressure) stimulates parasympathetic effects and inhibits sympathetic effects—resulting in decreased heart rate, vasodilation, and distribution of blood to skin and skeletal muscle. The neurons of the rostral ventrolateral nucleus are themselves sensitive to PO<sub>2</sub>, which shuts down a K<sup>+</sup> channel and causes an increase in the spontaneous discharge rate.<sup>10,11</sup> In addition, there is evidence that 1) nitric oxide (NO) acts as a neurotransmitter in the CNS, generally attenuating sympathetic tone by interacting with baroreceptor afferent inputs,<sup>12</sup> and 2) NO from S-nitrosothiol (SNO) adducts in red cells regulates the ventilatory response to hypoxia in the nucleus of the solitary tract.<sup>13</sup>

The carotid bodies also contain oxygen sensors that detect systemic hypoxia and quickly stimulate breathing and raise blood pressure via activation of the autonomic nervous system.<sup>14</sup> The O<sub>2</sub> chemoreceptor tissue in the carotid body includes two cell types. Type I, or glomus, cells are of neural crest origin and produce various neurotransmitters. Type II, or sustentacular, cells play a supportive role and resemble glial cells. It is thought that the Type I cells act as the O<sub>2</sub> sensors and trigger afferent nerve activation, which is transmitted to the CNS where it acts via autonomic control of respiratory rate and blood pressure. Although all cells respond to hypoxia, these responses usually evolve over a period of minutes to hours and require prolonged exposure to hypoxic conditions.<sup>15</sup> The carotid body, however, responds within seconds to decreases in the arterial PO<sub>2</sub> even as small as 20 mm Hg with excitation of the afferent nerves. This increased level of excitation is maintained if hypoxia persists and shows little adaptation.<sup>16</sup> The identities of the molecular elements of the O<sub>2</sub> sensor in Type I cells

are not clear, but candidates include several heme-containing proteins that use molecular O<sub>2</sub>, including cytochrome *a*<sub>3</sub>, nitric oxide synthase, heme oxygenase 2, and nicotinamide adenine dinucleotide phosphate oxidase.<sup>17–19</sup> Type I cells also express several O<sub>2</sub>-sensitive K<sup>+</sup> channel proteins which are inhibited by hypoxia.<sup>20,21</sup> There is evidence that multiple heme-containing proteins, may function as O<sub>2</sub> sensors, each sensitive to a different dynamic range of PO<sub>2</sub>. These proteins interact with O<sub>2</sub>-sensitive K<sup>+</sup> channel proteins (the “chemosome” hypothesis) to rapidly transduce the signal.<sup>22–24</sup> Hypoxia leads to depolarization of Type I cells and release of neurotransmitters. The major neurotransmitter that mediates afferent nerve activation by hypoxia is ATP. Paradoxically, ATP also acts on the Type I cells in an autocrine fashion to inhibit the hypoxia-stimulated increase in intracellular Ca<sup>++</sup> concentration,<sup>25,26</sup> and may release other inhibitory neurotransmitters as well.<sup>27,28</sup> It has been suggested that the release of both excitatory and inhibitory neurotransmitters functions as a “push-pull” mechanism to prevent over-excitation and signal extinction of the afferent nerves. This mechanism may also explain the lack of adaptation in the presence of persistent hypoxia.<sup>29</sup>

### Humoral Regulation of Blood Volume

Both atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) are produced in myocytes; ANP largely in the atria and BNP in the ventricle.<sup>30</sup> ANP is stored in perinuclear granules and released in response to increased atrial distension or pressure, as well as several neurotransmitters. BNP is not stored in granules but rather is synthesized and released episodically.<sup>31</sup> Volume expansion or pressure overload of the ventricular wall prompts synthesis of precursor forms of BNP that are processed and released.<sup>32</sup> Acting through receptor guanylate cyclases, these peptides have pleomorphic effects that generally contribute to peripheral vasodilation, natriuresis, and diuresis. BNP acts to inhibit the release of both antidiuretic hormone and aldosterone.<sup>33</sup> Acting in concert with sympathetic nerve input, it also has direct effects on the kidney, stimulating glomerular filtration, natriuresis, and diuresis.<sup>34,35</sup> In vascular smooth muscle cells, BNP, acting via the receptor guanylate cyclase and the Rho kinase system, stimulates dephosphorylation of myosin light chain, which promotes vasodilation.<sup>36</sup>

Blood volume is also regulated directly by the kidney. The renin axis is stimulated by a baroreceptor mechanism as well as the macula densa system. The cells of the macula densa respond to decreases in luminal NaCl concentration by stimulating the secretion of renin from the juxtaglomerular cells of the kidney. The secretion of renin initiates a proteolytic cascade beginning with the cleavage of angiotensinogen from the liver to produce angiotensin I. Angiotensin I itself is cleaved by angiotensin-converting enzymes (found in the lung and other tissues) to angiotensin II, and then angiotensin III. Both angiotensins II and III stimulate the secretion of aldosterone and thereby reduce diuresis.<sup>37</sup> The ability of the cells of the macula densa to sense NaCl levels depends on the function of the luminal  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  cotransporter,  $\text{NKCC2}$ .<sup>38,39</sup> The affinity of this cotransporter for  $\text{Cl}^-$  is low; hence, uptake is very sensitive to small changes in  $\text{Cl}^-$  concentration. Prostaglandins have been implicated as mediators of macula-dependent renin secretion,<sup>40</sup> partly on the basis of observations of impaired renin responses in the presence of COX-inhibitors and in COX-2 knockout mice.<sup>41-43</sup> Within the kidney, local production of endothelin activates NO secretion, which not only affects vasomotor tone and blood flow but also promotes sodium excretion as well.<sup>44</sup> In addition, increased intraluminal pressure is sensed by an as-yet-unidentified structure (possibly the juxtaglomerular cells themselves), which also acts through the release of prostacyclin ( $\text{PGI}_2$ ) to modulate renin release.<sup>45</sup>

Vascular volume is also regulated by the control of vasopressin over water metabolism. Arginine vasopressin (AVP) is synthesized as a prohormone in the magnocellular neurons in the supraoptic and paraventricular nuclei of the hypothalamus and transported to axon termini in the posterior pituitary gland.<sup>46</sup> There, the prohormone is processed to AVP and released constitutively at low levels. The magnocellular neurons receive input from cardiovascular control centers in the medulla that receive impulses from the baroreceptors of the carotid bodies and the aortic arch. AVP secretion is stimulated when blood pressure decreases, markedly so in hypovolemic shock. The magnocellular neurons also are innervated by osmoreceptor centers in the anterior hypothalamus that trigger AVP release when plasma osmolality increases. AVP release is also stimulated by prostaglandins, angiotensin II, and norepinephrine through  $\alpha_1$ -receptors, but inhibited by NO, ANP, and norepinephrine binding to  $\alpha_2$ - and  $\beta$ -receptors.<sup>47,48</sup>

AVP binds to a family of receptors on a variety of tissues including vascular smooth muscle and the kidney, where it exerts pressor and antidiuretic effects, respectively.<sup>49</sup> Binding of AVP to  $\text{V}_1$  receptors on vascular smooth muscle cells activates phospholipase C and stimulates the production of inositol triphosphate and diacylglycerol through the  $\text{G}_{q/11}$  pathway.<sup>50</sup> These second messengers in turn increase protein kinase C activity, leading to increased intracellular  $\text{Ca}^{++}$  concentration and contraction of the actin-myosin complex. AVP also binds to  $\text{V}_2$  receptors on the distal convoluted tubule and collecting ducts of the kidney where, via a cyclic adenosine monophosphate (cAMP)-dependent

pathway, it leads to insertion of Aquaporin-2 water channel proteins into the membrane of the luminal surface of the collecting ducts, enhancing the reabsorption of water.<sup>51</sup>

Although not as actively regulated as the above systems, blood volume is also influenced by the balance between the hydrostatic and oncotic forces at the capillary level. The movement of water and proteins across the capillaries provides for a relatively passive, but significant, mechanism for regulating intravascular volume.

### Erythropoiesis—Regulation of Oxygen Content

Oxygen delivery can also be enhanced by increasing the amount of oxygen delivered. This can be accomplished quickly through red cell transfusion, or physiologically, although more slowly, through erythropoiesis. The production of erythropoietin by cortical interstitial cells of the kidney in response to hypoxia is covered in more detail in Chapter 2. Upregulation of the transcription of the erythropoietin gene occurs rapidly after the onset of hypoxia, and is mediated by hypoxia inducible factor-1 $\alpha$  (HIF), which forms a transcription factor complex with other proteins.<sup>52</sup> Although the increase in erythropoietin production in response to hypoxia is prompt, the increase in the hematocrit is not seen for several days. Thus, this adaptation represents a long-term adjustment.

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## Regulation of Regional Oxygen Delivery

Blood flow to different organs is tailored to their levels of metabolic activity at rest, and actively altered to meet episodic physiologic needs, such as exercise or digestion. Blood flow is controlled by a mix of intrinsic and extrinsic systems. The local, intrinsic control mechanisms that operate in all organs facilitate the appropriate matching of oxygen delivery to metabolic need, while central control mechanisms make it possible to prioritize the distribution of blood flow among different organs and to preserve flow to critical organs such as the brain and heart. (See Table 3-2.)

### Coronary Circulation

Blood flow through the coronary circulation is greatest in the left ventricle, about half of that level in the right ventricle, and only a quarter of the left ventricular rate in the atria. Myocardial  $\text{O}_2$  consumption can increase by about five-fold during peak work, but is matched by a comparable increase in blood flow.<sup>53</sup> Even at rest, the myocardium extracts approximately 70% of the  $\text{O}_2$  delivered, which is much higher than any other resting tissue; thus, increased metabolic demands must be met by increased blood flow.<sup>54</sup> In the resting state, only about half of the myocardial capillary beds are open to perfusion in the resting state<sup>55</sup>; however, in hyperemia, additional capillary beds are recruited to augment flow.<sup>56</sup> Although the myocardial vasculature receives low-density innervation from the autonomic nervous system, blood flow is governed chiefly by intrinsic or metabolic regulatory mechanisms.<sup>57</sup> During sympathetic stimulation of heart rate and cardiac



**Table 3-2** Comparison of Oxygen Delivery to Selected Organs

| Organ or System | % of Cardiac Output | Oxygen Extraction                | Control                                 | Mechanism to Meet Demand                                           | Distinctive Features                                                                                   |
|-----------------|---------------------|----------------------------------|-----------------------------------------|--------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------|
| Coronary        | 5%                  | 70%                              | Intrinsic                               | Increased flow                                                     | Flow varies in systole/diastole; O <sub>2</sub> reserves depleted quickly                              |
| Cerebral        | 15%                 | 35%                              | Intrinsic (dominant) and some extrinsic | Controls flow to other organs to protect itself                    | Constant pH is critical; responsive to CO <sub>2</sub> levels                                          |
| Pulmonary       | 10%                 |                                  | Intrinsic and extrinsic                 |                                                                    | Hypoxic pulmonary vasoconstriction; low pressure                                                       |
| Skeletal/muscle | 20%                 | 25-30% at rest; to 90% on demand | Intrinsic and extrinsic                 | Arteriolar dilation; increased red cell flux through capillaries   | O <sub>2</sub> reserves depleted quickly with sustained vasoconstriction                               |
| Renal           | 20%                 |                                  | Intrinsic                               |                                                                    | High flow required                                                                                     |
| Splanchnic      | 24%                 |                                  | CNS; organ-specific factors             | Increased flow; opening of capillary beds previously underperfused | Flow can double for several hours after a meal; intense vasoconstriction during exercise or hemorrhage |

CNS = central nervous system

work, the weak sympathetic signal for vasoconstriction is overridden by intrinsic factors that increase blood flow to meet elevated O<sub>2</sub> demands. Similarly, while vagal release of acetylcholine would be expected to stimulate vasodilation, the decreased metabolic demands of the myocardium under parasympathetic influence leads to vasoconstriction, as intrinsic systems for matching blood flow and cardiac activity supersede autonomic input.

The nature of metabolic flow regulation of the coronary circulation is still unclear.<sup>58</sup> Several models have been proposed to account for the autoregulatory behavior of the coronary circulation. It was recognized early that hypoxemia and hypercapnia both produce vasodilation, and in a synergistic manner.<sup>59</sup> Prostaglandin-mediated vasodilation in response to low PO<sub>2</sub> levels has been shown to play a role in epicardial arterioles, but it is probably not a major factor in the regulation of coronary blood flow in hypoxia.<sup>60,61</sup> Hypercapnia (or the associated acidosis) has also been suggested to trigger autoregulatory responses from the arteriolar wall, or the subjacent myocardium via such mediators as adenosine, NO, or ATP-dependent K<sup>+</sup> channel activity.<sup>62</sup> However, while NO and ATP-dependent K<sup>+</sup> channel activity may contribute to vasomotor tone in the resting coronary arteries, none of these three mediators are responsible for local metabolic control during exercise.<sup>57</sup> It has also been hypothesized that release of ATP by red cells responding to hemoglobin desaturation<sup>63</sup> or NO released from S-nitroso-hemoglobin<sup>64</sup> might mediate a vasodilatory response in the coronary circulation.

The myocardial circulation is also unique in that flow varies with the mechanical activity of the heart, with most flow occurring during diastole; during systole, blood flow through the coronary capillaries comes to a virtual standstill. Blood flow is also unevenly distributed throughout the myocardium.<sup>65</sup> Compression forces are highest near the endomyocardium, and diminish progressively toward the epicardium.<sup>66</sup> To compensate, the internally located arterioles are more dilated than those nearer the epicardium. One consequence of this local

adaptation is that the subendomyocardial arterioles have less capacity to compensate for increased myocardial activity or anemia by further vasodilating, making this tissue particularly vulnerable to ischemia. The very high rate of O<sub>2</sub> utilization also makes the myocardium particularly sensitive to interruptions in blood flow. O<sub>2</sub> reserves are quickly depleted and within minutes the myocardium must switch to anaerobic glycolysis—thus generating lactic acid and, as ATP stores are drawn down and not replenished, inorganic phosphate. This further impairs muscle contraction. One protective feature of the myocardial circulation in some mammals, including humans, is the existence of collateral circulation, and the ability to stimulate its growth in response to decreased blood flow.

### Cerebral Circulation

About 15% of the cardiac output perfuses the brain, which, at rest, extracts approximately 35% of the delivered O<sub>2</sub>. Because gray matter has a much higher rate of oxidative metabolism than white, it receives a disproportionate fraction of the blood flow. The maintenance of ion homeostasis and membrane potentials is energy intensive and consumes a large proportion of the ATP generated by the neurons, which depend on a constant supply of O<sub>2</sub> and glucose. The O<sub>2</sub> reserves of the brain are very limited so that even brief periods of ischemia can produce hypoxia and irreversible cell damage because glycolysis cannot meet the energy demands of the neuron.

Cerebral blood flow is subject to both extrinsic and intrinsic regulation, although the latter is dominant, as is the case with the coronary circulation. Unlike other tissues, however, the brain also has the ability to control blood flow to other organs through autonomic output, and thus to protect its own blood supply. Extrinsic, autonomic innervation of resistance vessels in the brain has relatively weak effects on blood flow, and the blood-brain barrier excludes many humoral vasoactive molecules. Sympathetic input serves chiefly to shift the autoregulatory

curve to higher pressures, thereby protecting the brain from sympathetic-driven increases in blood pressure.<sup>67</sup> The parasympathetic system probably plays little part in autoregulation but has been implicated in ischemia and migraine.<sup>68</sup>

Cerebral resistance vessels and perivascular astrocytes both receive input directly from intrinsic CNS neurons (the neurovascular unit) located in the nucleus basalis, locus coeruleus, and raphe nucleus, which modulate vascular tone.<sup>67</sup> The projections of these intrinsic neurons contain acetylcholine, nitric oxide synthase (NOS), norepinephrine or 5-hydroxytryptamine, depending on their source. Perivascular astrocytes respond to these transmitters, as well as glutamate,  $\gamma$ -aminobutyric acid and ATP, and their endfeet are particularly rich in noradrenergic receptors, NOS,  $\text{Ca}^{++}$ -activated  $\text{K}^+$  channels, and purinergic receptors. These astrocytes release various vasoactive molecules such as ATP, adenosine, and NO, and exert both vasoconstrictive and vasodilatory influences via the release of 20-hydroxyeicosatetraenoic acid, or prostaglandin E2 and epoxyeicosatrienic acid, respectively.<sup>69</sup> The level of NO appears to influence which of these vasoactive molecules is produced. Astrocytes also release  $\text{K}^+$  via a  $\text{Ca}^{++}$ -activated channel that causes hyperpolarization of smooth muscle cells and may be responsible for their relaxation, although its role in functional hyperemia is not completely understood.<sup>70,71</sup>

The autoregulatory mechanisms of the brain maintain adequate cerebral blood flow over a wide range of arterial blood pressures (between 60 and 140 mmHg) and can adapt to even higher pressures in the face of chronic hypertension or acute sympathetic stimulation. These cerebral autoregulatory mechanisms also adapt to the considerable spatial and temporal heterogeneity of oxygen requirements<sup>72</sup> as reflected in the wide variation in tissue  $\text{PO}_2$  and hemoglobin  $\text{O}_2$  saturation throughout the brain demonstrated by studies using blood oxygen-dependent functional magnetic resonance imaging.<sup>73</sup> Increases in regional brain neural activity that increase metabolic demands trigger increased blood flow, probably by producing a temporary decrease in tissue  $\text{PO}_2$ .<sup>74-76</sup> Short-term responses to changes in cerebral  $\text{PO}_2$  appear to be mediated by  $\text{O}_2$ -sensitive  $\text{K}^+$  channels that are reversibly inhibited by hypoxia.<sup>77,78</sup> Gene transcription and protein synthesis may also be downregulated, the latter through inhibition of the function of eukaryotic initiation factors 1 and 2, as well as eukaryotic elongation factor 2,<sup>79-81</sup> thereby limiting the metabolic demands of the neurons.<sup>82</sup> Adaptation to persistent reduction in  $\text{O}_2$  supply requires changes in gene expression. Several  $\text{O}_2$ -sensitive transcription factors have been shown to respond to hypoxia, leading to changes in gene expression among them HIF, nuclear factor  $\kappa\text{B}$ , activator protein-1, and early growth response protein-1; however, the HIF system appears to play the central role in responses to changes in tissue  $\text{PO}_2$ . Among the scores of HIF-regulated genes are those that affect the regulation of vascular tone, angiogenesis, erythropoiesis, cell metabolism, and pH regulation.<sup>83</sup>

The cerebral circulation is also distinctive from others in its responsiveness to levels of  $\text{CO}_2$ . In fact, it appears to be even more sensitive to changes in arterial and tissue  $\text{PCO}_2$  than  $\text{PO}_2$ .<sup>84</sup>

Even small increases in the  $\text{P}_a\text{CO}_2$  stimulate vasodilation of cerebral arterioles.<sup>85</sup> Hypercapnic vasodilation may be mediated through effects on tissue pH, which decreases as the  $\text{PCO}_2$  rises. Because neuronal function is markedly impaired by increased  $\text{H}^+$  concentration, maintenance of constant pH is a critical process. There is also evidence that hypercapnia triggers closing of ATP-sensitive  $\text{K}^+$  channels.<sup>86</sup>

## Pulmonary Circulation

In contrast to the circulation through the brain and heart, the pulmonary circulation is a low-pressure, high-flow-rate system. Pulmonary pressures and resistances are only about a fifth of those found in the systemic circulation, although the entire blood volume circulates through the lungs approximately every minute. Ordinarily, only about 500 mL of blood are in the pulmonary vasculature. However, the vasculature can accommodate much larger volumes, particularly in the pulmonary veins, without a marked increase in resistance, thereby protecting the lungs from edema in the event of reduced left ventricular function or volume overload.<sup>87</sup>

Unlike the coronary and cerebral circulations, blood flow through the lungs is affected by both extrinsic and intrinsic factors and is highly responsive to its environment. Sympathetic innervation increases pulmonary vascular tone, although parasympathetic stimulation has little effect.<sup>88</sup> Some regulatory molecules also increase vascular tone, including angiotensin, serotonin, and prostaglandin F. Others, such as bradykinin and prostaglandin E1, reduce pulmonary resistance.

Hypoxic pulmonary vasoconstriction is a paradoxical but distinctive feature of the pulmonary vasculature.<sup>89</sup> Precapillary arterioles respond to the relative hypoxia of underventilated regions of the lung by constricting, thereby reducing flow and diverting it to better oxygenated alveoli. Pulmonary hypertension is prevented by the capacity of other vascular beds to absorb the diverted volume; however, widespread hypoxia may result in pulmonary hypertension.<sup>90</sup> The mechanism for hypoxic pulmonary vasoconstriction is not completely understood. Hypoxia has been shown to stimulate a small increase in the intracellular concentration of  $\text{Ca}^{++}$  in the smooth muscles of the pulmonary arteries. The effect of these small changes in  $\text{Ca}^{++}$  on smooth muscle actin-myosin contraction may be enhanced by "sensitizers" such as the Rho kinase signal transduction system.<sup>89,91</sup> It has also been observed that under conditions of hypoxia, NO consumption by red cells increases because of formation of hemoglobin  $\text{Fe}^{++}\text{NO}$ , which may potentiate other vasoconstrictive influences operative in this pulmonary reflex.<sup>92</sup> The pulmonary vasoconstrictive response to hypoxia is enhanced by metabolic acidosis<sup>93</sup> but muted by metabolic and respiratory alkalosis. This response is also decreased by various medications, including nitroprusside and some inhalational anesthetics, and in turn produces a decrease in the  $\text{P}_a\text{CO}_2$ .<sup>94</sup>

Circulation of blood through the lungs serves other purposes in addition to respiratory gas exchange with the alveoli and metabolic support of lung tissue. The lungs are also the site of the removal or inactivation of various vasoactive molecules

(eg, vasopressin, norepinephrine, acetylcholine), mediators of inflammation (eg, bradykinin, leukotrienes, prostaglandins E and F), and some medications (eg, propranolol, lidocaine, fentanyl) that in turn may have effects on vascular tone and blood flow. In addition, circulation of blood through the pulmonary bed serves to filter the venous return before its distribution through the arterial tree.

### **Skeletal Muscle Circulation**

At rest, the skeletal muscles of the body receive about 20% of the cardiac output. Skeletal muscle is heterogeneous, consisting of phasic fibers (glycolytic, rapid twitch) and tonic fibers (oxidative, postural). The majority of blood flow goes to the tonic fibers when the body is at rest, which is reflected in the higher density of the capillary beds around these muscle bundles. During exercise, however, the increased metabolic demands of the phasic fibers may be met with up to a 20-fold increase in blood flow.<sup>95,96</sup> Because most of the capillary beds are perfused at rest, increased flow is achieved through arteriolar dilation and increased red cell flux through the capillaries.<sup>97-99</sup> The hematocrit in the capillaries may rise from 10% to 15% at rest to 40% to 50% during hyperemia.<sup>99,100</sup> Given the striking ability to increase flow to actively exercising muscle, central cardiovascular control is required to prevent systemic hypotension and inadequate flow to other tissues.<sup>101,102</sup> Muscle can also extract more O<sub>2</sub>, increasing from a baseline oxygen extraction ratio of 25% to 30% to as much as 90%.

As is the case for the myocardium, blood flow in skeletal muscle is episodically interrupted by contraction and compression of the arterial vasculature, although red cells are still present in the capillaries during contraction and oxygen extraction continues, albeit at a reduced rate.<sup>103</sup> During periods of relaxation, the dilation of the arterioles and the opening of capillary beds also compensate for interruptions in flow. Indeed, there is evidence suggesting that contraction can activate a mechanosensitive response by the vascular endothelium or smooth muscle that stimulates vasodilation.<sup>104,105</sup> When contraction is sustained, however, tissue O<sub>2</sub> is quickly depleted, anaerobic glycolysis generates lactic acid, and ischemic pain is experienced.

Unlike the myocardium, blood flow to skeletal muscle is controlled by both extrinsic and intrinsic factors.<sup>106</sup> The arterioles in skeletal muscle are densely innervated with sympathetic nerves that maintain vascular tone and a tonic state of partial vasoconstriction. Arteriolar vasomotor tone is controlled centrally via reflex changes in autonomic output accompanying volition to exercise and in response to the arterial baroreflex. Additional sympathetic stimulation can produce greater vasoconstriction. In exercise, however, central autonomic sympathetic signals are overridden by intrinsic cues that trigger vasodilation. This occurs despite the fact that skeletal muscle also invokes the exercise pressor reflex, which augments blood pressure and heart rate through increased sympathetic outflow from the brainstem.<sup>107</sup> Mechanical<sup>108</sup> and/or chemical muscle receptors stimulated by muscle activity relay impulses to the dorsal horn of the spinal cord.<sup>109</sup> These impulses project onto many of the same nuclei in

the medulla that mediate the baroreflex pathway, among them the solitary tract nucleus and the rostral ventrolateral nucleus.<sup>110</sup>

Two other mechanisms have been implicated in the control of sympathetic output during exercise. Stimulation of the otoliths and vestibular system of the inner ear (by motion for example) enhances sympathetic activity of skeletal muscle and has been shown to produce vasoconstriction in the muscles of the forearm and calf.<sup>111,112</sup> In addition, elevated or reduced muscle temperature, respectively, increase or decrease sympathetic outflow during forearm exercise.<sup>113,114</sup>

### **Renal Circulation**

The kidneys receive about 20% of the cardiac output, a volume that considerably exceeds the flow required for perfusion. In part, the high flow reflects the metabolic demands of the kidney. Tubular reabsorption of sodium is an energy-intensive activity, consuming large amounts of O<sub>2</sub>. In addition, the kidneys require high flow to maintain hydrostatic pressure, which is the driving force for glomerular filtration. Renal blood flow is autoregulated to maintain a relatively constant rate of glomerular filtration over a wide range of arterial pressures by balancing the tone of the afferent and efferent arterioles serving the glomeruli. Two intrinsic systems interact to control blood flow through the kidneys.<sup>115</sup> The smooth muscle of the renal afferent arterioles responds to systemic arterial pressure by constricting when pressures increase and dilating as pressures decrease, ensuring constant blood flow. This myogenic response functions rather slowly, and does not compensate for fluctuations in systemic pressures of short duration (seconds).<sup>116</sup> The tubuloglomerular feedback mechanism is based on the macula densa responding to changes in blood flow with the release of vasoactive mediators, including adenosine, ATP, and NO, from the juxtaglomerular apparatus, in addition to renin as described above.<sup>117-119</sup> These vasoactive mediators affect local flow.

### **Splanchnic Circulation**

The circulation to the organs of the splanchnic bed is much more subject to central control than the circulation in other areas of the body. This circulation is characterized by dual control by organ-specific factors as well as the CNS. The arterioles of the splanchnic organs are particularly well innervated by sympathetic nerves but are also highly sensitive to the vasoconstrictors vasopressin and angiotensin II. These signals are capable of triggering intense vasoconstriction and reduction of flow under circumstances when blood must be diverted to other organs, as occurs in exercise or hemorrhage. In the absence of such demands, blood flow to the abdominal organs responds to local cues. Following meals, the metabolic needs of the digestive organs to support the secretion of acids and digestive enzymes or the adsorption and transport of nutrients, trigger increased blood flow to these tissues. This blood flow can double over a period of several hours following meals. The increased flow is largely achieved by opening capillary beds that were previously underperfused.<sup>120</sup> Gastrointestinal hormones and neuropeptides

released by cholinergic stimulation play a role in stimulating postprandial blood flow as do local mediators, including prostaglandins,<sup>121</sup> adenosine,<sup>122</sup> and NO.<sup>123</sup> Long chain fatty acids stimulate hyperemia of the proximal small intestine,<sup>124</sup> while bile acids evoke increased blood flow to the distal ileum.<sup>125</sup> Glucose has the most marked hyperemic effect of any of the nutrient stimulators of blood flow.<sup>126</sup>

The liver receives about 75% of its blood supply through the portal vein, which provides relatively deoxygenated blood. The remainder, which comes from the hepatic artery, is comparatively oxygen-rich. Blood flow from these two sources is controlled reciprocally to prevent congestion of the hepatic sinusoids, which could cause excessive filtration of water and plasma proteins through their highly permeable barriers.<sup>127</sup> A hyperdynamic splanchnic circulation with extensive portal-systemic shunting is typical of cirrhotic patients and plays a role in the development portal hypertension. The process is thought to be initiated by arterial vasodilation and has been related to NO overproduction.<sup>128</sup>

## Regulation of Oxygen Delivery in the Microcirculation

### The Microcirculation

The primary function of the microcirculation is to serve as a platform for the exchange of respiratory gases, nutrients, by-products of metabolic activity, and heat. The microvascular unit is composed of a reticulum of blood vessels with diameters less than 250 microns that lie between the arteries and the veins.<sup>1</sup> These beds of small vessels are dense in tissues with high metabolic activity and relatively sparse in tissues whose energy demands are modest. The arterioles arborize into a network of vessels that progressively decrease in caliber to form the terminal arterioles, with diameters of around 10 microns. The capillaries, which consist of a single layer of epithelial cells, have diameters of 3 to 10 microns, and drain into the postcapillary venules, and then to the venules.

Oxygen is delivered to the tissues by both convective and diffusive forces. Convection (blood flow) brings oxygenated blood to the microvascular unit. As oxygen is transferred from the incoming blood to the tissue, a longitudinal O<sub>2</sub> concentration gradient is created parallel to the direction of flow as more O<sub>2</sub> is extracted from the blood.<sup>129</sup> A second, radial, O<sub>2</sub> concentration gradient exists between the blood and the tissue surrounding the vessel, which is the driving force for O<sub>2</sub> diffusion into the tissue.<sup>130-132</sup> However, oxygen exchange in the microvascular unit involves more than the simple transfer of O<sub>2</sub> from red cell to tissue. Most O<sub>2</sub> is extracted from the blood at the level of the arterioles, particularly in skeletal muscle, with much of it being consumed by the arteriolar wall<sup>132</sup> to support smooth muscle activity and endothelial cell metabolism, including synthesis of NO, which requires molecular O<sub>2</sub>.<sup>133,134</sup> The O<sub>2</sub> concentration in the capillaries is generally near equilibrium with the surrounding

tissue so there is no gradient to drive O<sub>2</sub> into the tissues at that level.<sup>133</sup> The arterioles also serve as a source of O<sub>2</sub> to deoxygenated red cells flowing through capillaries<sup>135,136</sup> and the capillaries themselves exchange O<sub>2</sub> as well.<sup>136-138</sup> The extensive diffusion of O<sub>2</sub> among all of these elements of the microvascular unit is thought to be the basis for relatively homogeneous tissue oxygenation despite the considerable heterogeneity of blood flow through the capillaries.<sup>139</sup>

Under unstressed conditions, there is considerable temporal and spatial variability in blood flow through the capillary beds of the microcirculation. Depending on the tissue, only a quarter to a third of the capillaries are perfused at any time. When metabolic demands increase, however, a greater number of capillaries are perfused, increasing the effective area for O<sub>2</sub> exchange. Blood flow is actively regulated at the level of the resistance vessels as well as by changes in the vascular tone of the microvascular unit. Vasodilation is associated with decreased O<sub>2</sub> consumption by the arterial wall, which reflects decreased demand by smooth muscle, and thus a decreased O<sub>2</sub> gradient across the arterial wall.<sup>130</sup> More arterial O<sub>2</sub> is therefore available to the adjacent tissue.<sup>130,140-143</sup> A direct relation exists between intravascular PO<sub>2</sub>, arterial wall oxygen consumption, and microvascular tone.<sup>140,142,143</sup>

### Regulation of Microcirculatory Blood Flow

Blood flow through the microcirculation is controlled largely by intrinsic mechanisms, including pressure flow autoregulation, active hyperemia, and reactive hyperemia.<sup>144</sup> Pressure flow autoregulation refers to the ability of a tissue to maintain relatively constant blood flow over a wide range of systemic arterial blood pressures, vasodilating in response to elevated pressures and contracting when mean arterial pressure decreases. The effectiveness of this pressure-activated response is variable, but is generally robust in metabolically active tissues that must protect their oxygen supply. Active hyperemia is the ability of a tissue to meet metabolic demand by varying blood flow and the number of perfused capillaries, and is characteristic of tissue with episodic energy demands, such as skeletal muscle and the gastrointestinal tract.<sup>145</sup> Reactive hyperemia is the transient increase in blood flow, above baseline levels, which occurs after release of blood flow occlusion. The period of reactive hyperemia is usually proportional to the period of reduced flow, presumably reflecting the intensity of the hypoxic driver. All of these autoregulatory responses have been attributed to myogenic or metabolic mechanisms, although elements of both probably contribute to all of these phenomena.

The myogenic theory is based on the observation that vascular smooth muscle contracts when the tension or stretch of the arteriolar wall is increased and relaxes when tension drops. Smooth muscle cells respond to increased intraluminal pressure/stretch by depolarizing. This depolarization leads to activation of L-type voltage gated Ca<sup>++</sup> channels, Ca<sup>++</sup>-dependent activation of myosin light chain kinase, and actomyosin contraction.<sup>146</sup> This intrinsic, myogenic response explains pressure flow autoregulation and reactive hyperemia, but cannot account for active hyperemia, or responses to increased metabolic demand.

The metabolic theory is based on the coupling of flow to the level of  $O_2$  consumption of the tissue, and the observation that many metabolic by-products, such as  $CO_2$ ,  $O_2$ ,  $K^+$ ,  $H^+$  and adenosine, have vasoactive effects. According to this paradigm, tissue  $PO_2$  levels are read by an oxygen sensor of unknown identity. This sensor modulates the release of mediators of vascular tone including endothelin, NO, prostaglandins, and others. The sensor might be in the tissue itself, although the very rapid adjustments to flow and its sensitivity to intravascular  $PO_2$  are not entirely consistent with this location. It has also been suggested that the vessel walls—particularly the arterioles, which are the sites of  $O_2$  consumption and transfer—and/or red cells may serve as the sensors.<sup>147</sup>

Endothelial cells play a key role in integrating and conducting local vasoregulatory stimuli.<sup>148</sup> These cells act as signal transducers in their own right (eg, releasing NO in response to changes in shear stress)<sup>149</sup> but also serve as conduits to communicate signals along blood vessels through intercellular connections formed with adjacent endothelial cells.<sup>150</sup> Gap junction channels composed of connexins link endothelial cells to one another as well as to smooth muscle cells.<sup>151</sup> Six connexin subunits, proteins with four transmembrane-spanning domains, combine to form a hemichannel. Alignment of hemichannels on adjacent cells produces a complete gap junction. Through these connections, endothelial cells transmit signals as far as 1 mm, reaching resistance arterioles and influencing vasoactivity,<sup>152,153</sup> thereby accommodating increased flow through one capillary bed without diverting blood from another.<sup>148</sup> This property is called the conducted vasomotor response.

Endothelial cells exert their vasoregulatory effects through at least four mediators: endothelin, NO, prostaglandins, and endothelium-derived hyperpolarizing factor.

### Endothelin Regulation of Microcirculatory Blood Flow

Endothelin is a vasoconstrictor (usually) secreted by endothelial cells in response to a wide variety of signals.<sup>154</sup> Endothelial cells synthesize three isoforms of endothelin, of which endothelin-1 (ET-1) is the most important in terms of effect on vascular tone.<sup>155</sup> It is present in particularly large quantities in the pulmonary vasculature where its release is stimulated by hypoxia, but it is found throughout the vascular tree.<sup>156</sup> Endothelin is synthesized as preproendothelin-1 and then processed to big-ET-1 and ET-1 by ET-converting enzymes, a family of multiple metalloproteases.<sup>157</sup> ET-1 is secreted at low levels constitutively, primarily toward the basolateral side of the endothelial cells, and so acts principally as a local mediator rather than as a circulating hormone. ET-1 secretion is stimulated by vasoconstrictors (angiotensin II, vasopressin, norepinephrine), thrombin, several inflammatory cytokines, and physicochemical factors (low shear stress, hypoxia). Its release is inhibited by vasodilators such as NO, prostaglandins  $E_2$  and  $I_2$ , ANP, BNP, and bradykinin, as well as high levels of shear stress.<sup>158</sup>

ET-1 binds to one of two different G protein coupled receptor types found on the smooth muscle of blood vessels, the atrium,

and the ventricle. Binding of ET-1 to endothelin A receptors stimulates vasoconstriction via several signal transduction pathways.<sup>159</sup> Activation of the phospholipase C and 1,4,5-inositol triphosphate pathways elevates intracellular  $Ca^{++}$  concentration, which stimulates myosin light chain phosphorylation and contraction. ET-1 binding also inhibits myosin light chain phosphatase through activation of the Rho/Rho kinase pathway. Finally, ET-1 enhances phosphorylation of thin-filament associated protein through activation of c-Src, Janus tyrosine kinase, and protein tyrosine kinase, which stimulates extracellular signal-regulated kinases 1 and 2 (ERK1/2).<sup>160</sup>

Endothelin B receptors also mediate contraction of smooth muscle; however, they are found predominantly on endothelial cells.<sup>158</sup> When ET-1 binds to B receptors on endothelial cells, it stimulates release of NO and prostacyclin and produces a vasodilatory response in the adjacent smooth muscle. Exogenous endothelin administered to rats has been shown to have an initial vasodilatory (hypotensive) effect followed by a prolonged vasoconstrictive (hypertensive) effect, reflecting the dual nature of the A and B receptors.<sup>161</sup> ET-1 also stimulates production of vascular endothelial growth factor and potentiates the effects of platelet-derived growth factor and transforming growth factor- $\beta$ .

### Nitric Oxide Regulation of Microcirculatory Blood Flow

NO, or endothelium-derived relaxing factor, is synthesized from L-arginine and molecular oxygen by the enzyme NOS in a number of tissues but most notably in the vascular endothelium. The three isoforms of NOS (endothelial, inducible, and neuronal) are all activated in response to several mediators, including acetylcholine, bradykinin, ATP, and adenosine diphosphate (ADP), via G protein coupled receptors on the endothelial cell membrane that increase the level of intracellular  $Ca^{++}$ . NOS is a dimeric enzyme with a catalytic heme group and a zinc-thiolate domain, which is primarily structural.<sup>162</sup> It is localized principally in the caveolae of the plasmalemma and is inhibited by the major scaffold protein, caveolin-1, to which it is bound. When intracellular levels of  $Ca^{++}$  increase, calcium binds to calmodulin, which in turn displaces NOS and permits it to become catalytically active.<sup>163</sup>

In the vessel wall, NO released by endothelial cells diffuses to smooth muscle where it binds to guanylate cyclase coupled receptors and stimulates cyclic guanosine monophosphate (cGMP) synthesis.<sup>164,165</sup> The increase in cGMP level activates protein kinase G and produces muscle relaxation by opening  $K^+$  channels and/or decreasing the responsiveness of the contractile machinery to  $Ca^{++}$ ,<sup>166</sup> as well as modulating mitochondrial respiration.<sup>167</sup> Most vascular endothelial cells constitutively secrete low levels of NO, with the notable exceptions of the cerebral and coronary vessels.

In response to hypoxia, NO released by endothelial cells produces vasodilation, which results in redistribution of blood flow and the opening of more capillary beds.<sup>168</sup> Another factor influencing the production of NO (and endothelin) is shear stress, a property of a moving fluid that is influenced directly by its viscosity. Endothelial cells respond to decreases in shear stress

that occurs because of reduction of either flow velocity or blood viscosity (eg, anemia). In response to reduced shear stress, endothelial cells downregulate NO production and release, and increase endothelin release, resulting in vasoconstriction.<sup>169</sup> In animal models of hemorrhagic shock or isovolemic hemodilution, restoration of mean arterial pressure and capillary perfusion was better achieved with asanguinous fluids with higher viscosity compared to those with lower viscosity.<sup>170,171</sup> These effects were shown to be mediated by NO production based on direct measurement of NO by microelectrode and their abrogation by the presence of the NOS inhibitor N-nitro-L-arginine methyl ester, or in NOS-deficient mice.<sup>172</sup> In the same animal systems, equivalent restoration of microvascular flow was achieved with red cells whether they contained oxyhemoglobin or methemoglobin. These results suggest that the maintenance of blood viscosity, rather than oxygen delivery, was primarily responsible for successful maintenance of tissue perfusion.<sup>173,174</sup>

NO has wide-ranging effects beyond the vascular wall. In addition to its influence on vascular smooth muscle, NO also modulates systemic hemodynamic responses to hypovolemia, anemia, and hypoxia.<sup>175</sup> The vascular-dependent effects of NO on the heart include regulation of coronary tone, thrombogenicity, and angiogenesis. In addition, NO affects myocardial cell excitation-contraction coupling, mitochondrial respiration, and responses to autonomic input. Low levels of NO have a positive inotropic effect, while high levels have a negative inotropic effect. Negative chronotropic effects are mediated through the neuronal form of NOS in the cardiac ganglia, and the endothelial form in the myocardium.<sup>175</sup> NO released into the vascular lumen inhibits platelet aggregation and adhesion as well as leukocyte adhesion to the endothelium by interfering with the function of the CD11/CD18.<sup>162,176</sup>

### Prostaglandin Regulation of Microcirculatory Blood Flow

Prostaglandins are another set of molecular signals that endothelial cells release to modulate the activity of vascular smooth muscle. Arachidonic acid is released from the plasma membrane through the action of phospholipase A<sub>2</sub> and then metabolized to various eicosanoids by COX and other enzymes.<sup>177</sup> The COX-1 isoform is expressed in endothelial cells of the vascular wall and is responsible for a key step in the pathways that lead to the synthesis of both vasodilators [such as prostacyclin (PGI<sub>2</sub>), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), and prostaglandin D] and vasoconstrictors (such as prostaglandin F<sub>2</sub> and thromboxane). The major products in endothelial cells, however, are the vasodilators PGI<sub>2</sub> and PGE<sub>2</sub>. COX-2 is an inducible form of the enzyme that is also expressed by endothelial cells in inflammatory states and, in vitro, by prolonged periods (hours) of shear stress, stretch, hypoxia, or exposure to inflammatory cytokines, growth factors, or ET-1. Expression of COX-2 in response to these stimuli also leads to secretion of PGI<sub>2</sub>; however, it is not clear that there is a role for COX-2 in unstressed conditions.<sup>178</sup>

PGI<sub>2</sub> diffuses from the endothelial cell to the smooth muscle. The surface membrane of the smooth muscle cell expresses

a family of eicosanoid receptors.<sup>179</sup> The IP and EP<sub>2</sub> receptors for PGI<sub>2</sub> and PGE<sub>2</sub>, respectively, mediate vasodilation, whereas the TP receptors for thromboxane produce vasoconstriction. PGI<sub>2</sub> binding to the G-protein-coupled IP receptor activates adenylate kinase and increases cAMP levels. cAMP stimulates protein kinase A activity, which opens K<sup>+</sup> channels and causes membrane hyperpolarization; this, in turn, closes voltage-sensitive Ca<sup>++</sup> channels. The decrease in intracellular Ca<sup>++</sup> concentration results in relaxation of the smooth muscle cell and vasodilation.<sup>166</sup>

PGI<sub>2</sub> release is stimulated by bradykinin and hypoxia.<sup>178</sup> Conversely, hyperoxia has been shown to inhibit synthesis and release of vasodilator prostaglandins from endothelial cells and permit vasoconstriction, an effect that is independent of NO.<sup>180</sup>

### Endothelium-Derived Hyperpolarizing Factor in the Regulation of Microcirculatory Blood Flow

Endothelium-derived hyperpolarizing factor (EDHF) is a term used to define an as-yet-unidentified molecule (or process) that originates with the endothelial cell and produces smooth muscle relaxation, independently of NO or prostaglandins.<sup>152</sup> EDHF causes hyperpolarization of the smooth muscle membrane by activating K<sup>+</sup> channels that, in turn, close voltage-sensitive Ca<sup>++</sup> channels and elicit relaxation, as described for NO and PGI<sub>2</sub>. The mechanism whereby the endothelial cell produces this hyperpolarization of the smooth muscle cell membrane has not been completely worked out. The one step of the process that is well understood is the response of endothelial cells to agonists such as ATP. ATP increases the concentration of intracellular Ca<sup>++</sup> via activation of phospholipase C and inositol triphosphate-gated Ca<sup>++</sup> channels.<sup>181</sup> Several mechanisms have been proposed to explain how endothelial cells induce hyperpolarization of the smooth muscle cell membrane. One proposal is that the increase in endothelial cell intracellular Ca<sup>++</sup> leads to the synthesis and release of epoxyeicosatrienoic acids that open large-conductance, calcium-activated K<sup>+</sup> channels in the smooth muscle membrane, causing hyperpolarization.<sup>182,183</sup> K<sup>+</sup> itself, released from activated endothelial cells, may activate the inwardly rectifying K<sup>+</sup> channel (K<sub>ir</sub>) or Na/K ATPase in the smooth muscle cell, producing hyperpolarization.<sup>184,185</sup> It has also been suggested that the gap junctions between endothelial cells and smooth muscle cells either transmit the EDHF or propagate the hyperpolarizing current from cell to cell.<sup>186,187</sup> Finally, H<sub>2</sub>O<sub>2</sub> (produced by superoxide dismutase from the superoxide ion generated by endothelial cell NO) has been shown to activate several K<sup>+</sup> channels and elicit hyperpolarization.<sup>188</sup>

The physiologic role for EDHF is not completely understood.<sup>152</sup> EDHF-like dilations have been demonstrated in a wide variety of arteries and arterioles in vitro and in vivo. It is particularly prominent in smaller vessels, and may even supersede NO in some tissue beds. These observations suggest that EDHF may be a significant factor in regulating flow through the microcirculation. EDHF may also play a role in conducted vasomotor response, a property of microcirculatory beds that is

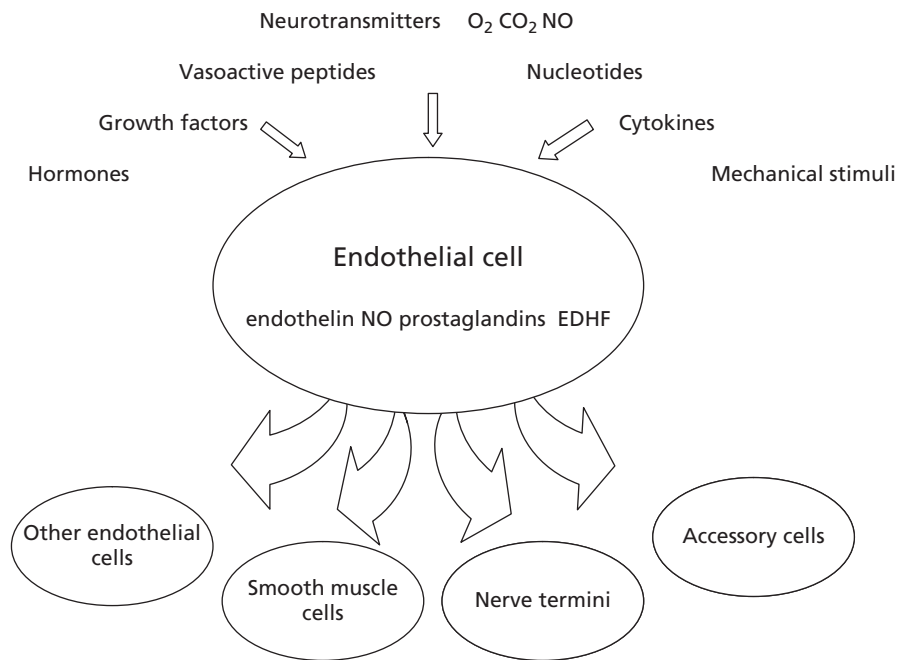


**Figure 3-1.** Endothelial cells receive inputs from various local and systemic sources, which they coordinate into a cohesive response with outputs to the smooth muscle cells of the vascular wall, other endothelial cells, and in some cases, nerve termini and other accessory cells. They play a central role in the regulation of blood flow and oxygen delivery through the microcirculation. NO = nitric oxide; EDHF = endothelium-derived hyperpolarizing factor.

retained even when the production of prostaglandin and NO are inhibited.<sup>189,190</sup>

### Coordination of Endothelial Control Mechanisms

Endothelial cells play an integrative role in the regulation of blood flow and the microvascular level (Fig 3-1). They are the recipients of myriad inputs including neurotransmitters, growth factors, hormones, vasoactive peptides, respiratory gases, nucleotides, and cytokines, as well as mechanical stimuli such as shear stress, stretch, and gravity. They are in physical contact with other endothelial cells, smooth muscle cells, nerve endings and, in some tissues, accessory cells such as astrocytes in the CNS. The endothelial cells also send out signals of their own, including endothelin, NO, prostaglandins, EDHF, adenosine,  $K^+$ , and others. The endothelial cell integrates all of this input and translates it into a coherent response that functions to adjust vascular tone. The relative contribution of the four main endothelial signals is not uniform through the vascular tree; NO serves as the major vasodilator in conduit arteries and EDHF is the major vasodilator in arterioles.<sup>191</sup> In addition, NO tonically inhibits EDHF responses as well as prostaglandin output. These complex interactions provide for a stable but responsive platform for oxygen delivery in the dynamic environment of the microcirculation.

### The Red Cell as a Regulator of Microcirculatory Blood Flow

It has been hypothesized that the red cell, or the hemoglobin therein, is a regulator of hypoxic vasodilation.<sup>192</sup> As hemoglobin deoxygenates, it undergoes a conformational change during the transition from the relaxed (R) to the tense (T) state; hence, it

could function as an oxygen sensor in the physiologically relevant  $PO_2$  range. Several models have been proposed to explain how the conformational change accompanying hemoglobin deoxygenation could be translated into a signal for vasodilation.

One model couples the R-T transition to release of ATP from the red cell, which then binds to purinergic receptors on endothelial cells, stimulating a vasodilatory response.<sup>193-196</sup> The model is supported by observations of increased levels of ATP in venous blood after hypoxia, the secretion of ATP from hypoxic red cells in vitro, the effect of ATP on vasomotor tone, and the retrograde propagation of the vasodilatory signal from the capillaries to the precapillary arterioles.<sup>194</sup> The conformation-induced release of ATP from red cells is mediated by the cystic fibrosis transmembrane conductance regulator.<sup>197</sup>

The second set of models proposes that the release of NO from the red cell in response to hemoglobin deoxygenation and the R-T transition is responsible for the vasodilatory effect. One mechanism of vasodilation is based on the release of NO from S-nitrosylated hemoglobin as it deoxygenates.<sup>198-200</sup> Although free NO has a very short half-life, NO adducts to cysteine thiols in proteins, while readily reversible, are quite stable. The S-nitrosothiol adducts can exchange with the free NO pool or other SNO adducts (transnitrosation) and in that manner can be transported far from the site of origin.<sup>201,202</sup> NO binds to the heme group of deoxygenated hemoglobin, but transfers to the  $\beta$ -93 cysteine upon reoxygenation<sup>202</sup> and has been shown to be released in hypoxia.<sup>203</sup> The anion exchange 1 macrocomplex may facilitate the exchange of gases, including NO, across the red cell membrane.<sup>204</sup> A number of experimental observations of the enhancement of hypoxic vasodilation in the presence of red cells have also been reported, consistent with a role in regulating blood flow.<sup>165,194,205-206</sup>

A second mechanism to explain NO release from red cells is based on the observation that hemoglobin has an intrinsic, allosterically regulated enzymatic activity that reduces nitrite anion ( $\text{NO}_2^-$ ) to NO.<sup>207-209</sup> Deoxyhemoglobin has more unliganded heme groups than the oxygenated form and can bind more  $\text{NO}_2^-$ ; however, the reduction rate constants are higher for oxyhemoglobin-mediated reduction. Maximal  $\text{NO}_2^-$  reduction therefore occurs where there is a mix of oxygenated and deoxygenated heme groups in the same molecule, which occurs around the  $P_{50}$ .<sup>209</sup> The reaction consumes a proton, hence hypoxia (or hemoglobin deoxygenation to levels of 40% to 60%; ie, around the  $P_{50}$ ) and acidosis would be expected to favor the generation of NO by this route. Exogenous  $\text{NO}_2^-$  has been shown to stimulate vasodilation in model systems,<sup>207-209</sup> experimental animals, and humans.<sup>210-213</sup> It is not clear if the  $\text{NO}_2^-$  pool operates as an independent source of NO, or in fact acts as a source of Fe bound NO, which is then transferred to  $\beta$ -93 cysteine and eventually released from the red cell.<sup>198,214</sup>

### The Microcirculation in Disease

As pointed out in Chapter 9, there is a critical point at which the rate of oxygen delivery is no longer adequate to support metabolic needs (oxygen consumption). It has been shown in stressed animals (eg, inflammation, sepsis) that this point occurs at higher ratios of  $\text{O}_2$  delivered to  $\text{O}_2$  consumed, even when the latter is not elevated, suggesting that the ability to extract  $\text{O}_2$  from the blood is impaired in these pathologic states. Inadequate tissue oxygenation when gross oxygen delivery is adequate may reflect disordered microcirculation.<sup>215</sup> Shunting of  $\text{O}_2$  from arterioles to venules has been described in sepsis and systemic inflammatory states, as has reduced tissue oxygenation when flow rates are high and red cell transit times through the arterioles and capillaries are too brief for effective  $\text{O}_2$  off-loading.<sup>216</sup> Shunting of  $\text{O}_2$ -rich blood around capillary beds also occurs when there is a decrease in the fraction of capillaries perfused, as has been demonstrated to occur in models of sepsis<sup>217-220</sup> and shock,<sup>221</sup> with increased  $\text{O}_2$  extraction by those capillaries that do remain open.<sup>222</sup> It has been suggested that the increased heterogeneity (maldistribution) of blood flow and resulting tissue hypoxia that occurs in sepsis and systemic inflammatory states is an early step leading to organ failure.<sup>221,223,224-227</sup> As a result, several approaches to microvascular resuscitation have been advocated combining volume replacement, inotropes, vasodilating drugs, and restrained use of vasopressors.<sup>228</sup> NO and prostacyclin in particular have been studied in human sepsis, and these vasodilators have been found to achieve some improvement in tissue perfusion and oxygen extraction.<sup>229,230</sup>

Capillary underperfusion in disease is thought to be the result of both vessel occlusion and impaired regulation of blood flow. One factor contributing to capillary occlusion is endothelial cell edema, which can be seen in sepsis.<sup>231</sup> In addition, the activated and immature leukocytes characteristic of systemic inflammation are less malleable and may mechanically occlude capillaries. Or, the leukocytes may interact with upregulated adhesion molecules on endothelial cell membranes, impeding

their progress through the microcirculation.<sup>232</sup> Red cells also exhibit reduced deformability in sepsis<sup>233-235</sup> perhaps related to overproduction of NO.<sup>234</sup> They also bind to endotoxin-exposed endothelial cells in vitro, suggesting that they, too, might occlude the microvasculature.<sup>236-238</sup>

Finally, disseminated intravascular coagulation could also occlude vessels with microthrombi.<sup>239</sup>

Arteriolar dysfunction also contributes to impaired blood distribution through the capillary beds in pathologic states. Arterioles exhibit abnormal responses to vasoregulatory signals in animal models of sepsis.<sup>240,241</sup> In addition, it has been shown that communication between endothelial cells is disrupted by lipopolysaccharide in vitro, and in animal model systems.<sup>241-244</sup> Impaired intercellular communication may be mediated by abnormal tyrosine phosphorylation of connexin 43, one of the gap junction proteins.<sup>245,246</sup> Excess NO production seen in inflammatory states is associated with endothelial cell and smooth muscle cell injury and may be responsible for defective arteriolar responses to regulatory cues, which results in the abnormal distribution of blood flow seen in these pathologic conditions.<sup>247</sup>

### Red Cell Transfusion and the Microcirculation

The effects of red cell transfusion on the microcirculation are just beginning to be understood. Direct assessment of microvascular function in patients is difficult, although minimally invasive techniques are becoming more widely available for this application. The analytic tools that have been used in the clinical setting include the  $\text{O}_2$  microelectrode, near infrared spectroscopy (NIRS), orthogonal polarizing spectroscopy (OPS), and blood oxygen-dependent function magnetic resonance imaging (BOLDfMRI).<sup>248</sup> The use of these techniques to assess the impact of red cell transfusion in humans has been limited.

#### $\text{PO}_2$ Microelectrodes

Direct measurements of tissue  $\text{PO}_2$  by microelectrode used in an observational study to assess brain tissue oxygenation in patients with subarachnoid hemorrhage or traumatic brain injury showed that the transfusion of one unit of Red Blood Cells (RBCs) increased tissue  $\text{PO}_2$  without changing the cerebral perfusion pressure.<sup>249</sup> However, transfusion of one or two RBC units to patients after coronary artery bypass grafting did not raise the  $\text{PO}_2$  in the deltoid muscle as measured by microelectrode.<sup>250</sup>

#### Near Infrared Spectroscopy

NIRS measures the degree of hemoglobin deoxygenation in the blood vessels in the sampled tissue and has been used widely as a monitoring tool in trauma,<sup>251,252</sup> sepsis,<sup>253,254</sup> brain injury and surgery,<sup>255,256</sup> and cardiac surgery.<sup>257-259</sup> NIRS has also been used to investigate the impact of blood loss on tissue oxygenation. Donation of 450 mL of whole blood was found to be associated with only a small decrease in cerebral blood oxygen saturation.<sup>260</sup> During removal of 740 mL of whole blood from research subjects, there was a correlation between  $\text{O}_2$  saturation levels measured in the cerebrum and calf muscle with the volume of



blood loss.<sup>261</sup> Similar results were obtained in acute normovolemic hemodilution.<sup>262</sup>

A few studies have investigated the effect of RBC transfusion on tissue oxygenation as assessed by NIRS. In a study in the neonatal intensive care unit (ICU), preterm neonates with symptomatic anemia were found to have higher fractional oxygen extraction, as determined by measurement of cerebral cortex tissue hemoglobin saturation by NIRS. When they received RBC transfusions, these infants showed a decrease in oxygen extraction to the levels seen in asymptomatic, anemic infants, and nonanemic infants.<sup>263</sup> NIRS monitoring of low-birthweight infants showed improvement in cerebral cortex oxygenation following RBC transfusion.<sup>264</sup> Both tissue oxygenation and cerebral blood flow improved when anemic, preterm infants received RBCs.<sup>265</sup> Intraoperative transfusion of 84 RBC units to 29 adult patients were monitored by NIRS. RBC transfusion was associated with improved cerebral and peripheral (calf muscle) oxygen saturation readings that correlated with the increase in hemoglobin level.<sup>266</sup> Decreases in cerebral blood oxygen saturation were noted in a high proportion of elderly patients undergoing abdominal surgery that correlated with the degree of blood loss; the decreases were correctable by RBC transfusion.<sup>267</sup> Notably, these changes in oxygenation were present in the absence of any decrease in blood pressure or other clinical signs of anemia.

### Orthogonal Polarizing Spectroscopy

Orthogonal polarizing spectroscopy assesses blood flow through the microcirculation.<sup>268,269</sup> It generates an image of the microcirculation based on the differential absorption and scattering of light, which distinguishes between patent blood vessels that contain red cells and vessels (including tissue) that do not. This technique can assess tissue perfusion in a manner that correlates with fractional capillary density. There are some limitations to OPS—chiefly, that the most suitable monitoring site in adults is the mucosa of the mouth (sublingual placement is common) and that it cannot be used on internal organs, except intraoperatively. It has been validated in animal systems and also against conventional capillary microscopy in human nail skinfold.<sup>270,271</sup> Altered microcirculation was demonstrated by OPS in patients with severe sepsis, and the changes were more severe in nonsurvivors.<sup>227,272,273</sup> Improvement in microcirculatory blood flow as assessed by OPS was also more strongly associated with good outcome than other changes in hemodynamic parameters or serum lactate levels.<sup>274</sup> Transfusion of 10 to 15 mL/kg of RBCs to anemic, preterm infants was shown to increase functional capillary density in blood vessels of the upper arm as measured by OPS.<sup>275</sup> The change was due to the perfusion of additional capillary beds because it was also shown that vessel diameter, red cell velocity, and flow rate did not change. Sublingual OPS was used to assess the effect of RBC transfusion in septic patients.<sup>276</sup> RBC transfusion resulted in increased hematocrit, mean arterial pressure, and calculated oxygen delivery; however, microvascular perfusion was unchanged, and was found to show considerable inter-patient variability.

### Blood Oxygen-Dependent Function Magnetic Resonance Imaging

BOLDfMRI detects changes in the ratio of oxyhemoglobin to deoxyhemoglobin. It has been used extensively to determine tissue oxygenation and blood flow in many tissues including brain,<sup>277</sup> kidney,<sup>278</sup> heart,<sup>279</sup> muscle,<sup>280</sup> and tumors.<sup>281</sup> It has not yet been used to assess hemorrhage or transfusion, but offers the advantage of being able to query any organ noninvasively.

### Animal Models

Direct assessment of the impact of RBC transfusion on the microcirculation has been studied more extensively in experimental animal systems. In a hemorrhagic shock model in rats, resuscitation with human red cells was associated with improved oxygenation of the mesenteric microcirculation as measured by O<sub>2</sub>-dependent quenching of palladium-porphyrin phosphorescence.<sup>282</sup> Similar results were obtained when this system was adapted to an isovolemic exchange model.<sup>283</sup> Experiments in the hamster skinfold system, where the microvascular circulation is visualized directly and can be coupled with O<sub>2</sub> level determination using palladium-porphyrin phosphorescence, showed better maintenance of microvascular blood flow and tissue oxygenation after exchange transfusion with fresh RBC units, than with units that had been stored for 28 days.<sup>284</sup> In a hemorrhagic shock model in the same hamster skinfold system, however, the same improvements in the microcirculation were obtained with fresh RBC units whether or not they contained functional hemoglobin or methemoglobin, suggesting that the rheologic effects of RBC transfusion were more important than the ability of the red cells to off-load O<sub>2</sub>.<sup>174,285</sup>

### Effect of RBC Storage Changes on the Microcirculation

During storage, RBC units undergo several changes. It has been suggested that these changes affect their ability to deliver O<sub>2</sub>. Although there has been considerable interest in defining the clinical impact of these storage changes, clinical studies and experiments in animal model systems have yielded conflicting results. Several observational studies in cardiac surgery, trauma, and ICU patients have shown an association between prolonged storage time and poor clinical outcomes<sup>284,286-290</sup>; however, several other studies, including a pilot randomized, controlled trial in ICU patients, have not found deleterious effects after the transfusion of longer storage RBC units.<sup>277,276,291-294</sup> The effect of the storage changes on the microcirculation is also being evaluated.

Among the characteristic changes that red cells undergo during storage is alteration of their shape from a biconcave disc to an echinocyte with protrusions from which lipid vesicles are shed. This shape change is associated with loss of deformability<sup>295,296</sup> and increased osmotic fragility that results in hemoglobin loss to the plasma-anticoagulant-preservative phase.<sup>297</sup> Given the ability of hemoglobin to scavenge NO, RBC units with free hemoglobin could trigger vasoconstriction and impede blood flow. In fact, there is evidence that hemoglobin scavenging of NO contributes to vaso-occlusive crisis in sickle cell disease.<sup>298</sup> Reduced red cell deformability might also impede flow as a result of impaired

transit through small vessels, although this effect has been shown to be mitigated by the shunting of rigid or misformed red cells to larger diameter vessels in the microcirculation.<sup>299</sup> Stored red cells have been shown to adhere to endothelial cell layers in vitro and to small vessels in rat muscle, suggesting another mechanism whereby transfused red cells could occlude the microcirculation.<sup>300</sup> Candidate adhesion molecules include the Lutheran and LW antigens,<sup>301</sup> and the thrombospondin receptor, CD36.<sup>300</sup>

Levels of 2,3-DPG decrease during red cell storage. The loss of this allosteric modifier is reflected in a decrease in the hemoglobin  $P_{50}$  from 29 mm Hg to approximately 20 mm Hg. After transfusion, the red cell replenishes its 2,3-DPG over a period of 24 hours.<sup>302</sup> Increased  $O_2$  affinity has been suggested to impair oxygen off-loading. No effect of experimentally altered  $P_{50}$  on global parameters of oxygen dynamics or the microcirculation was apparent in several studies in experimental animals.<sup>303–305</sup> Conversely, in three studies in rats, tissue oxygenation (as assessed by palladium-porphyrin phosphorescence or microelectrode) and/or functional capillary density (as assessed by intravital microscopy) showed that tissue oxygenation and capillary perfusion were not as well maintained by transfusion of RBC units that had been stored for various periods and were presumably deficient in 2,3-DPG.<sup>283,306,307</sup> Transfusion of red cells from mice with a low-affinity hemoglobin variant (hemoglobin Presbyterian) conferred a survival advantage in a mouse LPS-challenge sepsis model.<sup>308</sup> The behavior of hemoglobin-based oxygen carriers (HBOCs) is different than that of intraerythrocytic hemoglobin. In two animal model systems, high-affinity hemoglobin preparations preserved functional capillary density and tissue oxygenation better than preparations formulated with lower-affinity hemoglobin.<sup>309,310</sup> High oxygen affinity is thought to balance the enhanced propensity of the HBOCs, which are much smaller than red cells, to facilitate diffusion of  $O_2$  to the vessel wall and thereby avoid triggering autoregulatory vasoconstriction and impaired blood flow through the microcirculation.<sup>311</sup>

ATP levels in stored RBC units decrease to around 60% of initial levels after 5 weeks of storage, and are restored a few hours after transfusion, a process that involves the uptake of adenosine.<sup>312</sup> ATP levels do correlate with posttransfusion red cell survival, but only at levels below 50%, which is usually reached only after the standard storage period of 42 days.<sup>313</sup> However, restoration of ATP levels in stored RBC units improved tissue oxygenation in a model system,<sup>283</sup> suggesting that ATP plays a role, whether as an energy source for a metabolic process or as a vasodilator, in oxygen delivery.

It has recently been observed that red cell levels of SNO-hemoglobin decline rapidly during storage, decreasing by 70% within a day of collection.<sup>314</sup> These SNO-depleted red cells failed to induce vasodilation in isolated rabbit aorta rings or to promote blood flow through canine coronary arteries in vivo; however, these properties could be restored by re-nitrosolating the red cells by exposure to NO in solution. This change may explain, in part, the experimental observations of the failure of RBC transfusion to improve global and/or microcirculatory parameters of oxygenation.

## Red Cell Substitutes

Investigators have long sought a substitute for red cells that would successfully imitate its oxygen delivery capabilities. Interest in the development of materials that could be used to deliver  $O_2$  to tissues in place of red cells was initially stimulated in the 1980s by concerns about the adequacy of the blood supply and transfusion-transmitted infectious diseases. This interest has been sustained primarily because of the clinical potential for an effective red cell substitute and secondarily because of the insights into oxygen delivery and its regulation that have been gained through the study of these materials.<sup>315–317</sup> The search for a red cell substitute has led to the investigation of three different classes of oxygen carriers: modified hemoglobin, perfluorocarbon emulsions, and liposome-encapsulated hemoglobin. The development of the latter remains in the preclinical stage,<sup>318</sup> but several perfluorocarbon- and hemoglobin-based substitutes have advanced to the level of Phase III trials.

### Hemoglobin-Based Oxygen Carriers

Cell-free hemoglobin is an obvious candidate for a red cell substitute and has a number of desirable attributes, the first of which is its ability to carry high concentrations of  $O_2$  and  $CO_2$ . The absence of the red cell membrane eliminates the complications created by the presence of blood group antigens. The purification procedures remove other cell components that could produce undesirable effects, and a variety of processes are used to eliminate or inactivate infectious agents. In its purified form, hemoglobin solutions are quite stable and can be stored for much longer periods than RBC units and under more permissive conditions (eg, room temperature). However, hemoglobin is a highly active molecule, and in its unmodified state, has the potential of producing a number of deleterious effects.<sup>319</sup> These toxicities have been responsible for the failure of several candidate oxygen carriers in clinical trials and were recently addressed at a symposium sponsored by the National Heart, Lung, and Blood Institute. The symposium identified areas of particular concern requiring more research to sustain future development.<sup>320</sup> In an effort to eliminate or mitigate some of these potential toxicities, all of the HBOCs being studied make use of hemoglobin preparations that have been chemically modified.

One problem with free hemoglobin in plasma is that it dissociates into  $\alpha\beta$  dimers and is rapidly cleared by glomerular filtration. Not only does the hemoglobin leave the bloodstream too rapidly to be clinically useful, but also it is toxic to the renal tubular cells.<sup>321</sup> Various modifications have been made to all HBOCs under study; these changes have had the effect of increasing the plasma half-life and eliminating nephrotoxicity. Three types of modifications have been used to achieve these ends: intramolecular stabilization of the hemoglobin tetramer; intermolecular cross-linking (polymerization of the tetramer); and surface conjugation of hemoglobin with other macromolecules such as polyethylene glycol (PEG).

A second concern with the hemoglobin molecule is its vasoactivity. The hypertensive effects of cell-free hemoglobin have been observed in animals<sup>322,323</sup> and humans<sup>324</sup> and are attributed, in part, to NO scavenging by the heme iron and the resulting vasoconstriction.<sup>325</sup> In addition, the HBOCs are not S-nitrosylated, like hemoglobin in red cells, and therefore lack the vasodilatory activity of the intact red cell.<sup>326</sup> However, in-vitro S-nitrosylation of a pegylated hemoglobin was recently shown to enhance its ability to promote vasodilation of the coronary arteries in dogs undergoing open-chest surgery.<sup>327</sup> Other factors that play a role in the vasoactivity of HBOCs are the effects of their low viscosity<sup>328</sup> on vascular tone.<sup>329,330</sup> Delivery of O<sub>2</sub> by the substitute may also exert local vasoconstrictive effects by triggering an O<sub>2</sub>-sensitive autoregulatory response.<sup>331,332</sup> The degree of vasoactivity varies among the different HBOC compounds and is generally more prominent in preparations based on a hemoglobin species with small molecular radius (eg, stabilized hemoglobin tetramer) compared to a species with a large radius (eg, pegylated hemoglobin).<sup>333</sup>

Hemoglobin in solution auto-oxidizes to methemoglobin, which does not carry O<sub>2</sub>, thence to irreversibly altered metabolites including free radicals,<sup>334</sup> which could have deleterious effects on tissues exposed to them. Methemoglobin formation has been measured in all of the HBOCs studied to date and found to be <15% in all, with little tendency to accumulate during storage.<sup>335</sup> The possibility that HBOCs might be immunogenic is low but is being assessed in the various clinical trials.<sup>336</sup> Human hemoglobin is a poor immunogen, but chemically modified human hemoglobin, or bovine hemoglobin, which has 90% sequence homology with the human form, could conceivably be more highly immunogenic. Surface modification with macromolecules such as PEG and polyoxyethylene may shield hemoglobin from the immune system and reduce the frequency or magnitude of the response.<sup>337</sup>

The HBOCs under development have been based on hemoglobin from three sources: human, from outdated donor RBC units; bovine; and recombinant technology. Seven HBOCs, four of them based on human hemoglobin, have been studied in clinical trials, as have two HBOCs based on bovine hemoglobin and one using a recombinant human hemoglobin. (See Table 3-3.) Only three remain under active development, although a newer product based on lyophilized human hemoglobin, OxyVita (OxyVita, New Windsor, NY), is in preclinical testing.<sup>338</sup>

PolyHeme (Northfield Laboratories, Evanston, IL) is a preparation consisting of a glutaraldehyde polymerized human hemoglobin that has been pyridoxylated and extensively purified to remove residual hemoglobin tetramers.<sup>339</sup> (See Table 3-4.) It is being developed as an alternative to RBC use in surgery and trauma.<sup>340,341</sup> Trauma patients who received PolyHeme required fewer transfusions of banked blood.<sup>340</sup> Northfield recently completed a pivotal Phase III clinical trial of the use of PolyHeme as an early intervention in trauma.<sup>342</sup> The study was controversial for its waiver of individual informed consent, which has occasionally been used in clinical studies in trauma patients, as well as for the

study designs, which called for continued use of the study material even after patients arrived at the hospital and could have received banked blood. Although there was a trend for excess 1-day and 30-day mortality in the treatment arm, the differences were not statistically significant. Myocardial infarction was reported more frequently in patients receiving PolyHeme ( $p < 0.05$ ).

The Biopure Corporation (Cambridge, MA) uses glutaraldehyde to polymerize bovine hemoglobin followed by extensive purification and virus inactivation steps to produce a substitute with less than 3% hemoglobin tetramers (Hemopure). The Biopure Corporation is the first to have obtained licensure for a blood substitute product. In 1997, a veterinary formulation of their HBOC, Oxyglobin, was licensed by the Food and Drug Administration (FDA) for use in animals and in 2001, Biopure announced that Hemopure had been approved for clinical use in South Africa. This product has been studied principally for perioperative use as a “bridge,” delaying the need for banked RBCs until the patient has been stabilized and blood loss curtailed.<sup>343-345</sup> Biopure has reported in abstract form on the safety and efficacy of Hemopure in Phase II and III trials in orthopedic surgery, where it was shown to reduce the use of allogeneic RBCs<sup>346</sup>; however, the firm has announced that it is in the process of designing several Phase II trials in nonsurgical applications.<sup>347</sup>

Hemospan (Sangart, San Diego, CA) is a pegylated human hemoglobin designed specifically with a large molecular radius and high oxygen affinity to mitigate the diffusive transfer of O<sub>2</sub>, which is thought to trigger the vascular autoregulatory response responsible for the vasoactivity characteristic of many of the other HBOCs.<sup>310,348,349</sup> This product has undergone Phase I and II clinical testing in Europe where it was found to have minimal hypertensive effects.<sup>350,351</sup> A Phase II clinical trial in patients undergoing radical retropubic prostatectomy is being mounted in the United States and a Phase III trial in orthopedic surgery is open in Europe.<sup>352</sup>

### Perfluorocarbon-Based Oxygen Carriers

The perfluorocarbons (PFCs) are a class of compounds consisting of linear or cyclic carbon backbones that are highly substituted with fluorine.<sup>353</sup> Most of the PFCs are liquid at room temperature and are highly soluble for respiratory gases including O<sub>2</sub>, N<sub>2</sub>, and CO<sub>2</sub>. The amount of gas that can be dissolved into a PFC is directly proportional to its ambient partial pressure. Theoretically, at a high enough PO<sub>2</sub>, a PFC could contain the same amount of O<sub>2</sub> as blood. PFCs are not miscible in water, however, and must be prepared as emulsions for most biologic applications. The need to prepare the PFCs as emulsions decreases the O<sub>2</sub>-carrying capacity; therefore, patients must receive supplemental oxygen when a PFC emulsion is used.

PFC emulsions are cleared by the reticuloendothelial system (RES), as are the HBOCs, but eventually are exhaled via the lungs. The half-lives in the RES of some of the first PFCs studied were quite prolonged, but the newer PFCs have half-lives in the RES on the order of a few days. The impact on the RES of the ingestion of large amounts of PFCs or the emulsifying

**Table 3-3.** Modified Hemoglobin-Based Red Cell Substitutes Reaching Clinical Trials\*

| Product Class                          | Modification                                   | Product (Manufacturer)                         | Hemoglobin Source | Trial Level                          | Application                                                                                                                                |
|----------------------------------------|------------------------------------------------|------------------------------------------------|-------------------|--------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------|
| Intramolecular cross-linked hemoglobin | Di-aspirin                                     | HemAssist (Baxter)                             | Human             | Phase II                             | Septic shock, hemodialysis, hemorrhagic shock, cardiopulmonary bypass                                                                      |
|                                        |                                                |                                                |                   | Phase III (discontinued)             | Acute blood loss—surgery, trauma                                                                                                           |
|                                        | Recombinant di- $\alpha$ hemoglobin            | Optro (Somatogen Baxter)                       | Recombinant       | Phase I                              | Stroke                                                                                                                                     |
|                                        |                                                |                                                |                   |                                      | Erythropoiesis—ESRD, refractory anemia                                                                                                     |
| Polymerized hemoglobin                 |                                                |                                                |                   | Phase II (discontinued)              | ANH, acute blood loss, surgery                                                                                                             |
|                                        |                                                |                                                | Human             | Phase III                            | Trauma, surgery                                                                                                                            |
|                                        | Glutaraldehyde polymerization + pyridoxylation | PolyHeme (Northfield)                          |                   |                                      |                                                                                                                                            |
|                                        | Glutaraldehyde polymerization                  | Hemopure <sup>†</sup> (Biopure)                | Bovine            | Preclinical<br>Phase I<br>Phase II   | Erythropoiesis<br>Radiosensitizer, glioblastoma<br>Sickle cell crisis, oncology, surgery—orthopedic, urological, vascular, cardiac, trauma |
| Surface conjugation                    |                                                |                                                |                   | Phase III (on hold)<br>Approved      | Surgery—cardiac, orthopedic<br>Veterinary—anemia, acute blood loss                                                                         |
|                                        | Glutaraldehyde polymerization o-rafifnose      | Oxyglobin (Biopure)                            | Bovine            |                                      |                                                                                                                                            |
|                                        |                                                | Hemolink (Hemosol)                             | Human             | Phase II                             | Cardiopulmonary bypass—ANH, orthopedic surgery, acute blood loss, dialysis                                                                 |
|                                        |                                                |                                                |                   | Phase III (discontinued)             | Cardiac surgery                                                                                                                            |
| Surface conjugation                    | PEG                                            | Hemospan (Sangart)                             | Human             | Phase II<br>Phase III                | Prostatectomy, orthopedic surgery<br>Orthopedic surgery                                                                                    |
|                                        | Polyoxyethylene PEG                            | PHP (Ajinomoto/Apex)<br>PEG Hemoglobin (Enzon) | Human<br>Bovine   | Phase III<br>Phase Ib (discontinued) | NO-induced shock<br>Radiosensitizer solid tumors                                                                                           |

\* Information current as of May 2008.

<sup>†</sup> Approved in South Africa.

ESRD = end-stage renal disease; ANH = acute normovolemic hemodilution; PEG = polyethylene glycol; PHP = polyoxalated hemoglobin polyoxyethylene; NO = nitric oxide.

**Table 3-4.** Characteristics of HBOCs in Phase III Clinical Trials

| Characteristic                   | Hemoglobin-Based Oxygen Carriers        |                |           |
|----------------------------------|-----------------------------------------|----------------|-----------|
|                                  | PolyHeme                                | Hemopure       | Hemospan  |
| Hemoglobin source                | Human                                   | Bovine         | Human     |
| Modification                     | Glutaraldehyde<br>pyridoxal-5-phosphate | Glutaraldehyde | PEGylated |
| Molecular weight (kD)            | 150                                     | 240            | 96        |
| Molecular radius (nm)            | 2.7                                     | 4.9            | 14.1      |
| % tetramer (64 kD)               | <1%                                     | <3%            | 100%      |
| P <sub>50</sub> (mm Hg)          | 28-30                                   | 38             | 6 ± 2     |
| % Methemoglobin                  | <3%                                     | <3%            | <5%       |
| Hemoglobin (g/dL)                | 10                                      | 13             | 4.2       |
| Colloid oncotic pressure (mm Hg) | 20-25                                   | 17             | 55        |
| Change in mean arterial pressure | ↔                                       | ↑ 15%          | ↔         |

**Table 3-5.** Perfluorocarbon Red Cell Substitutes Reaching Clinical Trials\*

| Product (Manufacturer)         | Perfluorocarbon              | Trial Level                 | Application                             |
|--------------------------------|------------------------------|-----------------------------|-----------------------------------------|
| Fluosol-DA (Green Cross/Alpha) | Perfluorodecalin             | Phase II (discontinued)     | Acute blood loss                        |
|                                | Perfluoropropylamine         | Approved (withdrawn)        | PTCA                                    |
| Oxygent (Alliance)             | Perflubron                   | Phase II                    | CABG—ANH Ortho surgery—acute blood loss |
|                                |                              | Phase III (discontinued)    | Cardiac surgery, surgery—ANH            |
| Imagent GI (Alliance)          | Perflubron                   | Approved (withdrawn)        | GI imaging                              |
| Imavist (Alliance)             | Perflubron                   | Phase III (discontinued)    | Cardiac ultrasound                      |
| Liquivent (Alliance)           | Perflubron (neat)            | Phase Ib/II                 | Liquid ventilation—IRDS                 |
|                                |                              | Phase II/III (discontinued) | Liquid ventilation—pedi and adult ARDS  |
| Oxyfluor (HemaGen/PFC)         | Perfluorodichlorooctane      | Phase II (discontinued)     | Surgery, neuro-protectant/bypass        |
| Oxycyte (Synthetic Blood Int.) | Perfluoro-t-butylcyclohexane | Phase IIb                   | Acute brain injury                      |

\*Information current as of May 2008.

PCTA = percutaneous transluminal coronary angioplasty; CABG = coronary artery bypass graft; ANH = acute normovolemic hemodilution; GI = gastrointestinal; IRDS = infant respiratory distress syndrome; ARDS = acute respiratory distress syndrome.

agents remains to be determined. The clearance rate of PFC emulsions from the circulation increases as the emulsion particle sizes decrease. Thus, reproducibly and predictably preparing emulsions with the appropriate distribution of particle sizes is one of the technical challenges that the developers of these materials have faced. The plasma half-lives of the PFC emulsions that have been studied for clinical use are approximately 12 to 24 hours, which compare favorably with the half-lives of the HBOCs.

Two PFC emulsions underwent clinical trials in the 1990s but development of both has been discontinued. (See Table 3-5.) Two new products are in preclinical or clinical testing. One, PherO<sub>2</sub> (Sanguine, Pasadena, CA) is a reformulation of perfluorodecalin, one of the constituent PFCs in Fluosol, a product licensed in the 1980s for use in percutaneous transluminal coronary angioplasty to perfuse the vascular bed distal to the balloon, but which saw off-label use as a transfusion equivalent.<sup>354</sup> Synthetic Blood

International (Costa Mesa, CA) announced that its PFC product, Oxycyte, was more effective at increasing brain oxygen levels in patients with traumatic brain injury than either 50% or 100% O<sub>2</sub>. The firm has announced plans for a Phase IIb study in 200 patients with traumatic brain injury.<sup>355</sup>

### Red Cell Substitutes: Summary

Although the various preparations that have been studied share the ability to transport oxygen, they are quite distinct from one another and certainly very different from red cells. If the compounds are to be used to best advantage, transfusion medicine specialists will need to be familiar with the capabilities and liabilities of the substitutes that find their way into the clinical arena. The development of these substitutes has shed considerable light on the mechanisms of oxygen delivery and its regulation, and forced reconsideration of some long-held assumptions. In order to adequately fulfill the role of consultant, the transfusion

medicine specialist will need an even greater understanding of the physiology of red cell transfusion and oxygen delivery.

## Conclusion

A constant supply of oxygen to the tissues is essential to sustain the basic energy-producing machinery of the cell. Homeothermic organisms have evolved multiple, interlinked regulatory systems to ensure that oxygen delivery to the periphery meets the specific metabolic requirements of each tissue and is coordinated with the function of other systems.

## Disclaimer

The author has disclosed no conflicts of interest.

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# 4

## Red Cell Metabolism and Preservation

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A human red cell, mature and released from the marrow, lacks a nucleus and mitochondria. It has a life span of 120 days, after which it is removed from the circulation in the natural course of aging. It can neither use oxygen for the extraction of energy, nor synthesize proteins or nucleotides. Its primary functions—transporting oxygen from the lungs to the tissues and carrying carbon dioxide back to the lungs—do not require the expenditure of energy. However, maintaining hemoglobin in an optimal state for delivery of oxygen and keeping a normal cell morphology do require active metabolism, and are prerequisites for function and successful transfusion.

During standard storage at 4°C, significant and, in part, reversible changes in red cell morphology and metabolism occur. One striking example is the change in red cell shape (Fig 4-1), which can be reversed to a large extent by metabolic rejuvenation of the cells. In order to optimize storage with the development of optimal strategies, it is essential to understand basic principles of red cell metabolism.

Another example of a reversible change is the binding of oxygen. As detailed in Chapter 3, hemoglobin flips between a taut (low oxygen affinity) state (T) and a relaxed (high oxygen affinity) state (R). Therefore, the process of oxygenation causes the hemoglobin molecule, like the lungs, to “breathe.” However, oxygenation and deoxygenation of hemoglobin are not isolated events, because parallel to the intrasubunit and tetrameric conformational movements related to the T and the R states are changes in the binding of hemoglobin to its other ligands [hydrogen ions, carbon dioxide, chloride ions, 2,3-diphosphoglycerate (2,3-DPG), band 3 in the cell membrane, and nitric oxide]. Together with the influence of temperature, these ligands are mutually interactive in determining the conformational state of hemoglobin, and thus its oxygen affinity.

Results obtained in the past decade by several research groups indicate that many intermolecular interactions may be

missed when hemoglobin is considered by itself without taking into account the cellular milieu in which it performs its main biological function.<sup>2,3</sup> Thus, interaction of hemoglobin and band 3 is influenced by conformational changes that accompany ligand binding to hemoglobin, which modulates the function of band 3, the glycolytic pathway, and the structural architecture of the red cell membrane-cytoskeletal system (Fig 4-2). Human red cells move rapidly through the tissue and lung capillaries (transit time of 0.3-1 second). Cells in the tissue surrounding the microcapillary endothelium signal their requirement for oxygen by release of carbon dioxide and protons. To satisfy this requirement, oxygen must be released from red cells in the vicinity of the cells that signal their need for it.<sup>3</sup> Thus, red cell carbon dioxide uptake and oxygen release must be completed within the brief time during which the red cell traverses the capillary and is in contact with endothelial cells. Moreover, the close contact of hemoglobin, band 3, and carbonic anhydrase in the cell membrane promotes the production of bicarbonate with release of protons. The release of protons via the Bohr effect (see Chapter 3) in turn releases oxygen from hemoglobin where it is needed in the tissues. In the following section, red cell metabolism is discussed taking into account intermolecular interactions of importance for the primary function of hemoglobin, which is the transport of oxygen and carbon dioxide.

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### Metabolism

#### Metabolism of Glucose

Under physiologic circumstances, the energy that the red cell requires is derived through the breakdown of glucose to lactate or pyruvate. The sequence of reactions is generally known as the glycolytic or the Embden-Meyerhof pathway.<sup>4</sup> This pathway is phylogenetically very old, and the sequence of reactions is the same in bacteria, yeast, and vertebrates. Except for the exaggerated production of 2,3-DPG (also known as 2,3-bisphosphoglycerate) in the red cell, the pathway is the same in all tissues.



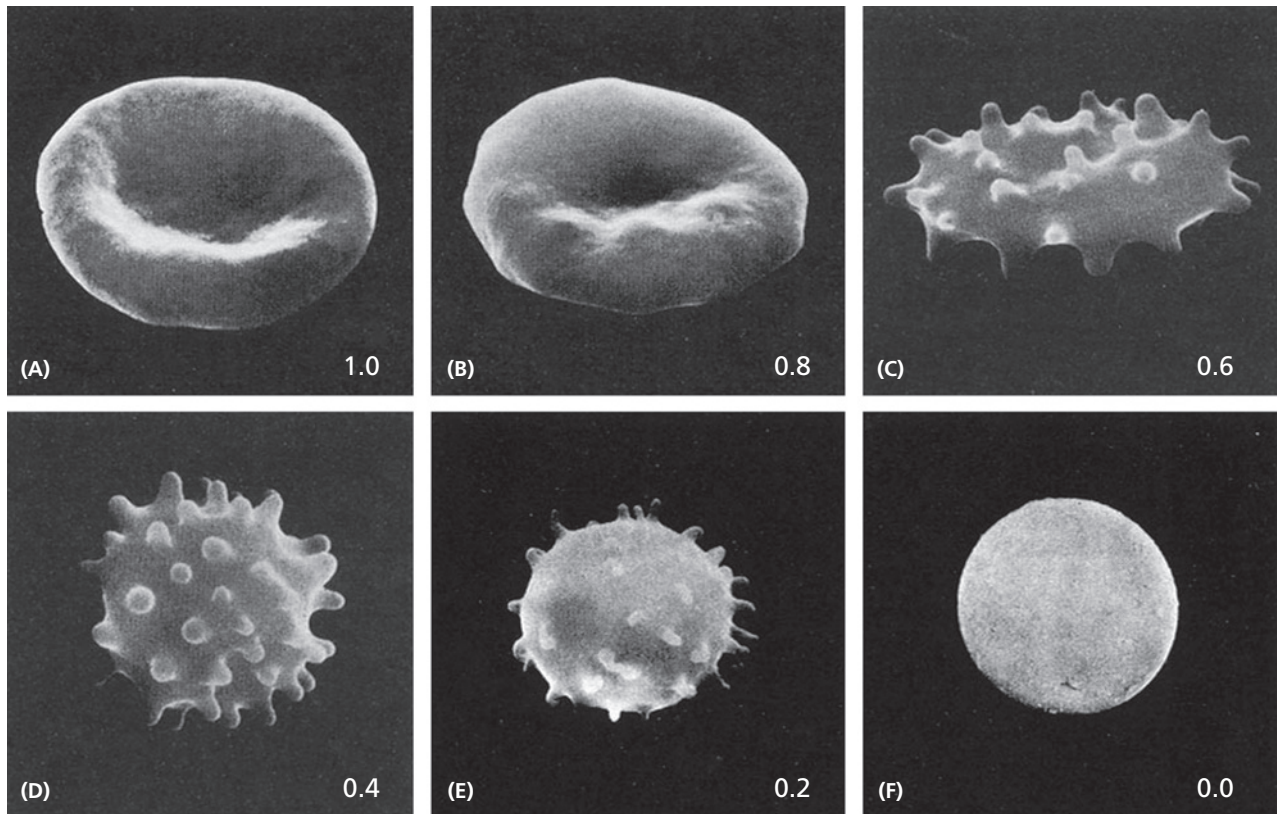
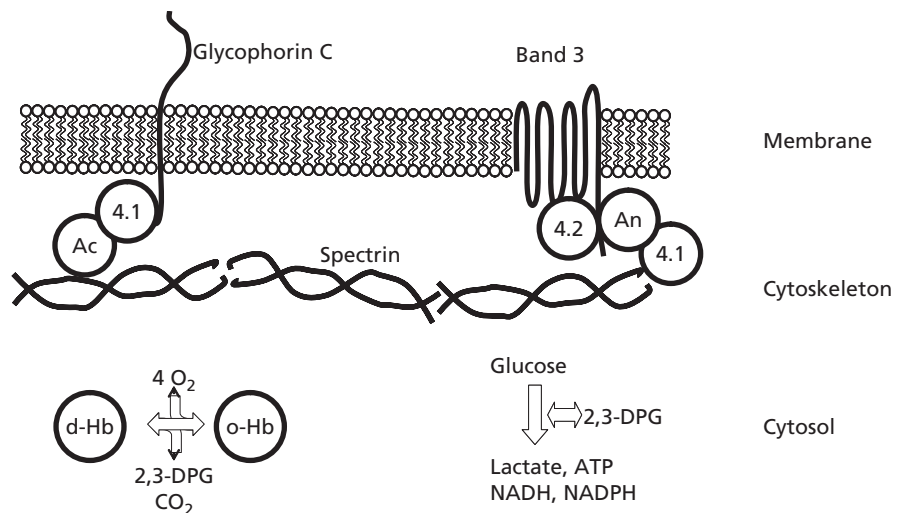


**Figure 4-1.** Scanning electron micrographs showing representative red cells in various stages of shape change typical of prolonged storage.<sup>1</sup> The cells progress from discocytes (A), through several stages of echinocytes (B-D), to spherocytes (E), and finally to spherocytes (F). Scores based on the visual appearance (shown in the lower right of the individual images) can be assigned to several hundred individual stored cells and averaged to produce a morphology score for the unit. Such scores decrease in a linear manner during storage but can be substantially reversed by rejuvenation.

**Figure 4-2.** Schemata of red cell components.

In the past, it was believed that oxygen transport by hemoglobin and metabolism were cytosolic functions, and that the membrane and cytoskeleton merely enclosed them. It is now recognized that the attachments of the membrane to the cytoskeleton from band 3, through ankyrin (An) and proteins 4.2 and 4.1 to spectrin, and from glycophorin C through protein 4.1 and actin (Ac) to spectrin are destabilized by 2,3-diphosphoglycerate (2,3-DPG). 2,3-DPG released from deoxyhemoglobin (d-Hb) binds to band 3 and partially detaches the membrane from the cytoskeleton, allowing lateral movement of membrane structures. This could have implications for red cell flexibility in peripheral capillaries. Other abbreviations: O-Hb = oxygen-hemoglobin.



The reactions of the glycolytic pathway are shown in Fig 4-3. In a sequence of reactions, the six-carbon sugar glucose is phosphorylated, isomerized to fructose phosphate, phosphorylated again, and cleaved into three-carbon sugars. The three-carbon sugars are again phosphorylated. Finally, the carbohydrate-bound phosphate that has been gained is transferred to adenosine

diphosphate (ADP), producing the high-energy compound, adenosine triphosphate (ATP). The synthesized ATP is used by ATPases for the pumping of ions against concentration gradients, for the phosphorylation of membrane proteins and lipids, and very importantly for the phosphorylation of glucose in the glycolytic pathway.<sup>5</sup>



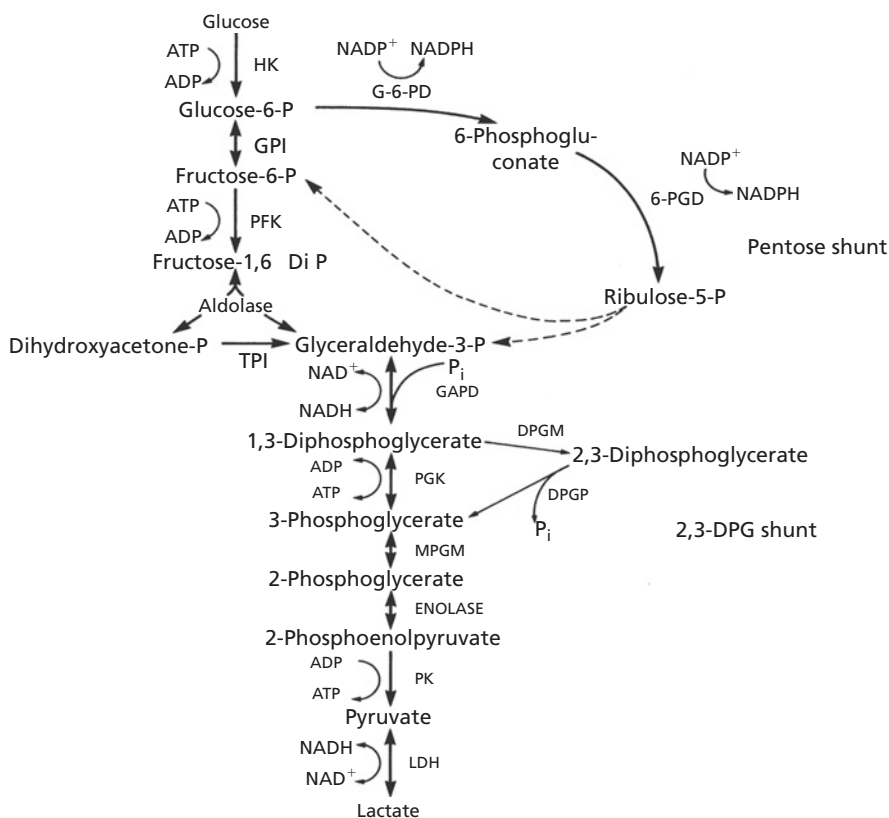


**Figure 4-3.** The glycolytic pathway with the pentose and 2,3-DPG shunts of red cell metabolism. ATP = adenosine triphosphate; ADP = adenosine diphosphate; HK = hexokinase; NADP<sup>+</sup> = nicotinamide adenine dinucleotide phosphate, oxidized form; NADPH = nicotinamide adenine dinucleotide phosphate, reduced form; 6-PGD = 6-phosphogluconate dehydrogenase; P = phosphate; G-6-PD = glucose-6-phosphate dehydrogenase; PFK = phosphofructokinase; TPI = triosephosphate isomerase; NAD<sup>+</sup> = nicotinamide adenine dinucleotide, oxidized form; NADH = nicotinamide adenine dinucleotide, reduced form; GAPD = glyceraldehyde-3-phosphate dehydrogenase; PGK = phosphoglycerate kinase; DPGM = diphosphoglycerate mutase; DPGP = diphosphoglycerate phosphatase; MPGM = monophosphoglycerate mutase; PK = pyruvate kinase; LDH = lactate dehydrogenase.

### The 2,3-DPG Shunt (Rapoport-Luebering Shunt)

Production of 2,3-DPG is the function of a shunt that branches from the main glycolytic pathway after the formation of 1,3-diphosphoglycerate (1,3-DPG) and returns to it with the formation of 3-phosphoglycerate (3-PGA) (Fig 4-3). The pathway consists of the formation of 2,3-DPG from 1,3-DPG, followed by the dephosphorylation of 2,3-DPG to 3-PGA (Fig 4-4). Both reactions are catalyzed by the same enzyme and are balanced at physiologic pH.<sup>6</sup> At higher pH, the enzyme acts only as a mutase, moving phosphate in 1,3-DPG from position 1 to position 2 in the molecule; at low pH it acts only as a phosphatase, transforming 2,3-DPG to 3-PGA. As the 2,3-DPG shunt bypasses one of the two ATP-making steps in glycolysis, 2,3-DPG is made at the expense of ATP. In storage systems, a high pH can shut down ATP production, while a lower than physiologic pH leads to a burst of ATP production driven by the breakdown of 2,3-DPG.

The production of large quantities of 2,3-DPG is a unique feature of glycolysis in the red cell. Red cells contain approximately equimolar amounts of hemoglobin and 2,3-DPG. Binding of 2,3-DPG to the  $\beta$  subunits of deoxyhemoglobin serves to stabilize the T (low oxygen affinity) state of hemoglobin and thus shifts the oxygen equilibrium curve to the right (favoring dissociation of oxygen). In the R (high oxygen affinity) state, approximately 80% of 2,3-DPG is “free,” while in the T state over 80% of 2,3-DPG is bound to hemoglobin.<sup>7</sup> In the “free” state 2,3-DPG at physiologic concentrations modulates

properties of the red cell membrane.<sup>2</sup> It binds directly to band 3 and thereby interferes negatively in the interactions between protein 4.1, 4.2, ankyrin, and band 3.<sup>8</sup> In addition, 2,3-DPG releases spectrin from the membrane skeleton, and interferes negatively in the interactions between spectrin-actin-protein 4.1 and the glycophorin C complex.<sup>2,9,10</sup> This decreases the number of connecting links between the cell membrane and the cytoskeleton and increases lateral mobility of integral membrane proteins.<sup>11</sup> The rise and fall of 2,3-DPG concentrations with each pass through the circulatory system, therefore, results in repetitive destabilization and restabilization of the membrane-cytoskeleton architecture (Fig 4-2). “Free” 2,3-DPG increases cell flexibility by weakening the links between the membrane and the cytoskeleton, and facilitates gas exchange by allowing the red cell to slip into narrow capillaries and splenic sinusoids. However, further experiments are needed to clarify the physiologic implications of the interactions of 2,3-DPG, cell membrane proteins, and the cytoskeleton.

### The Pentose Shunt (Hexose Monophosphate Shunt)

Under normal, steady-state conditions, most glucose is metabolized in red cells by way of the glycolytic pathway, but there is another important metabolic pathway called the pentose shunt or hexose monophosphate shunt (Fig 4-3). Some of the glucose-6-phosphate (G-6-P) formed when glucose is phosphorylated in the hexokinase reaction may enter this pathway. Glucose-6-phosphate dehydrogenase (G-6-PD)

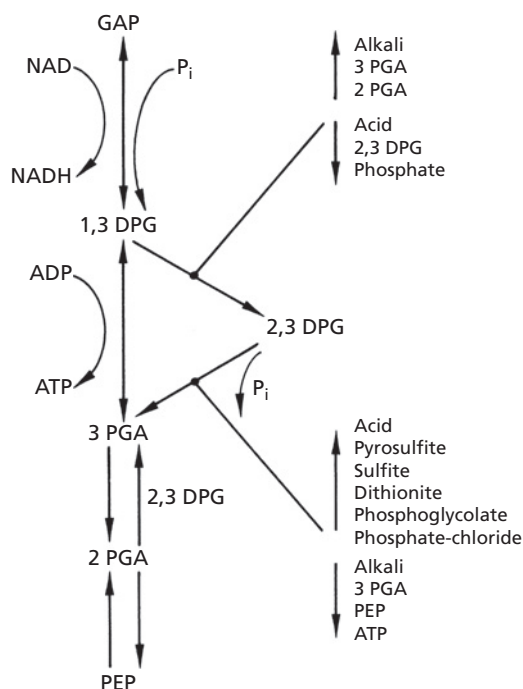


**Figure 4-4.** Regulation of the formation and breakdown of 2,3-DPG. The steady-state concentration of 2,3-DPG is governed by the rate of its formation from 1,3-diphosphoglycerate and by its breakdown to 3-phosphoglycerate. Both reactions are catalyzed by the same multifunctional enzyme modulated by a variety of substances. Formation of 2,3 DPG by the mutase (DPGM) is enhanced by substances to the right of the upward arrow, and reduced by those to the right of the downward arrow. Similarly, arrows indicate substances modulating the breakdown of 2,3 DPG by the phosphatase (DPGP). GAP = glyceraldehyde-3-P; 3 PGA = 3-phosphoglycerate; 2 PGA = 2-phosphoglycerate; PEP = 2-phosphoenolpyruvate.

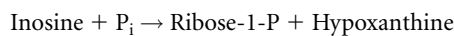
catalyzes the oxidation of G-6-P to 6-phosphogluconolactone, reducing nicotinamide adenine dinucleotide phosphate (NADP) to NADPH. After hydrolysis of the lactone to 6-phosphogluconic acid, another oxidative step reduces additional NADP to NADPH, and releases carbon dioxide from the six-carbon compound, forming the pentose sugar ribose-1-phosphate. After a series of rearrangements, two normal intermediates of the main glycolytic pathway—fructose-6-phosphate and glyceraldehyde-3-phosphate—are formed and rejoin the main metabolic stream.

The pentose shunt is important to the red cell primarily as a source of NADPH. It is this reduced nucleotide that maintains glutathione (GSH) in its reduced form. Reduced GSH is important for the elimination of peroxide, protection of protein sulfhydryl (SH) groups and detoxification processes. The pentose shunt also plays an important role for the red cell by providing ribose-5-phosphate needed for the production of phosphoribosyl pyrophosphate (PRPP), an essential substrate for the synthesis of adenine nucleotides required for ATP synthesis (see below).

### Alternative Substrates for Red Cell Metabolism

Glucose is the natural substrate for human red cell energy metabolism, but red cells are also capable of metabolizing other sugars (ie, fructose, mannose, galactose, and the three-carbon sugar dihydroxyacetone). However, none of these other sugars have proven to be useful in the design of blood preservatives.

The presence in red cells of the enzyme nucleoside phosphorylase makes it possible for red cells to use nucleosides such as inosine to support ATP synthesis:



In this reaction, ribose-1-phosphate is formed without the expenditure of ATP. Ribose-1-phosphate is then readily converted to fructose-6-phosphate by the pentose shunt, which feeds into the glycolytic pathway leading to the generation of ATP. In the rejuvenation of red cells (see later) inosine allows ATP-depleted red cells to prime their metabolic pump (the glycolytic pathway). It has not been possible to include inosine in blood preservatives because the product of its metabolism, hypoxanthine, is rapidly converted to uric acid in the body. Because many patients who receive blood transfusions have impaired liver function or may already have hyperuricemia because of hereditary or acquired factors, a blood component that increases plasma uric acid level cannot be considered safe.<sup>12</sup>

### Regulation of Energy Metabolism

#### Rate of Glucose Metabolism

In general, metabolic regulation is dependent on protein synthesis, which, in turn, is regulated by increasing or decreasing the rate of DNA transcription or the translation of messenger RNA. Red cells do not have this option. Instead, the rate of glucose metabolism is regulated by feedback mechanisms. However, in spite of extensive studies, our understanding of the control of glucose metabolism by red cells is still incomplete.

The N-terminal cytoplasmic domain of band 3 binds hemoglobin, cytoskeletal proteins, and glycolytic enzymes.<sup>13</sup> Based on current evidence, de Rosa et al<sup>2</sup> point out that interaction between hemoglobin in the T state and band 3 causes a release of glycolytic enzymes, which results in increased activity of the main glycolytic pathway. On the other hand, hemoglobin in the R (highly oxygenated) state is associated with increased activity of the pentose shunt because the enzymes of the main glycolytic pathway show reduced activity when bound to band 3.<sup>14</sup>

The rate of glucose metabolism by red cells is influenced by many factors other than enzyme activity. Negative feedback mechanisms seem to be involved in the glycolytic pathway and 2,3-DPG is involved in several. Both hexokinase and phosphofructokinase are inhibited by hydrogen ions. Thus, one of the principal reasons for the markedly slow rate of glycolysis during blood storage is the accumulation of lactic acid in whole blood or the acidity of first-generation additive solutions.

### 2,3-DPG Concentration

The concentration of 2,3-DPG depends on the rate of its formation and degradation. Many effectors determine whether the mutase or the phosphatase activity of the diphosphoglycerate mutase/phosphatase predominates (Fig 4-4). The hydrogen ion concentration is one of the most important physiologic modulators. At low pH, phosphatase activity is stimulated and mutase activity is inhibited. Thus, high pH favors 2,3-DPG maintenance and production during storage at the expense of ATP formation, whereas low pH leads to the rapid loss of 2,3-DPG with an increase in ATP production. Modulation of red cell metabolism by pH has been the principal means used to retard the decline of 2,3-DPG levels that occurs during liquid storage of red cells (see later). After depletion during storage, 2,3-DPG levels in transfused red cells return to 50% of normal in 7 hours and to almost 95% at 72 hours.<sup>15</sup>

### Pentose Shunt Activity

The rate of the pentose pathway is influenced by the availability of NADP and the level of NADPH. Under oxidative stress NADPH is oxidized to NADP and the activity of the pentose pathway increases, which is consistent with the observation of increased pentose shunt activity when hemoglobin is in the R state.

### Adenosine Triphosphate

In the red cells, ATP is synthesized by the glycolytic pathway, but its regulation is complex. ATP is used in a number of different metabolic pathways—particularly by the kinases, which phosphorylate sugars (ie, hexokinase, phosphofructokinase) and proteins, and by ATPase-driven ion pumps (ie,  $\text{Na}^+/\text{K}^+$ -ATPase,  $\text{Mg}^{2+}$ -ATPase, and  $\text{Ca}^{2+}$ -ATPase). Moreover, red cell membrane shape and rigidity are controlled by the ATP-dependent cytoskeleton.

ATP is in equilibrium with ADP and adenosine monophosphate (AMP). As the level of ADP increases, some is converted to AMP. AMP, in turn, is deaminated in the AMP-deaminase reaction. The total red cell pool of adenine decreases during storage, which leads to the depletion of ATP if adenine is not added to the anticoagulant and/or the additive solution.

### Guanine Nucleotides

Red cells contain and turn over guanine nucleotides, which perform at least two functions in red cells. First, guanine nucleotides play a role in the signal transduction of membrane shear into secretion of local vasodilators cyclic AMP and ATP.<sup>16</sup> Second, high concentrations of guanosine triphosphate (GTP) inhibit red cell transglutaminase, a primitive coagulation system that also interacts with the cytoplasmic domain of band 3 and with protein 4.1.<sup>17</sup> The GTP concentration is reduced when red cells age so that transglutaminase, which has a Factor XIII-like activity, can facilitate the removal of senescent red cells by binding them to fibrin clots.

### Synthetic Processes

Red cells are able to assemble only a limited repertoire of important molecules from simpler precursors. They retain the capacity to synthesize nucleotides through a “salvage pathway” and to

synthesize GSH from its precursor amino acids. Adenine is able to enter the erythrocyte and purine nucleotides are synthesized through the adenine phosphoribosyl transferase reaction:



This reaction is critical in blood storage. The beneficial effect of addition of adenine to stored blood depends on it. PRPP, one of the substrates for the formation of AMP in this reaction, is synthesized from pentose-phosphate formed in the pentose shunt. Guanine nucleotides form in an analogous reaction and are catalyzed by a different enzyme, hypoxanthine-guanine phosphoribosyl transferase. Red cells also actively synthesize a number of other small molecules, including reduced glutathione, NAD, and S-adenosyl-L-methione.<sup>5</sup>

### Membrane Metabolism

The red cell membrane is composed of a phospholipid bilayer containing cholesterol molecules and membrane proteins.<sup>18</sup> The phospholipids are disposed with a predominance of phosphatidylcholine and sphingomyelin in the outer leaflet and phosphatidylinositol, phosphatidylethanolamine, and phosphatidylserine (PS) in the inner leaflet. Loss of phospholipid asymmetry results in exposure of PS, which is an important apoptotic marker on the red cell surface. Exposure of PS on the outer cell surface promotes red cell removal from the circulation, while the surface glycoprotein CD47, which decreases during storage, inhibits phagocytosis.<sup>19</sup> The phospholipid asymmetry is maintained by the ATP-dependent flippase (aminophospholipid translocase) activity. This activity counteracts phospholipid scrambling, which moves PS from the inner to the outer cell surface. Flippase activity decreases during storage, but can be corrected by rejuvenation of the red cells.<sup>20</sup> Phospholipid scrambling is normally low during storage, but can be enhanced by photodynamic treatment for pathogen inactivation.<sup>21</sup>

Maintaining the iron of hemoglobin in a reduced state is a prerequisite for effective oxygen transport. The enzyme involved is methemoglobin reductase, which reduces  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  by oxidizing the reduced form of nicotinamide adenine dinucleotide (NADH) to NAD. Protection of the SH groups of hemoglobin and membrane proteins from oxidation is also a crucial function. This is accomplished by maintaining adequate amounts of reduced GSH by oxidation of NADPH to NADP. The oxidation of GSH to GSSG catalyzed by peroxidase reduces  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$  and  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ . Adequate levels of ATP, NADH, NADPH, and 2,3-DPG for these metabolic functions are secured by the glycolytic pathway with its pentose and 2,3-DPG shunts. In addition, the synthesis of NAD and NADP from nicotinic acid must also occur.

Membrane proteins such as glycophorin and band 3 extend through the lipid membrane. Branches of carbohydrates anchored to membrane proteins protrude from the outer surface of the membrane. The inner surface of the membrane is lined by the cytoskeleton, which is anchored to the membrane through the N-terminal cytoplasmic domain of band 3. The tetrameric form of band 3 binds ankyrin and constitutes with proteins 4.1

and 4.2 a major attachment site for spectrin. A second attachment site for spectrin involves actin and protein 4.1 in the glycoporphin C complex. Both attachments (Fig 4-2) are weakened when the concentration of “free” (non-hemoglobin-bound) 2,3-DPG is increased; this could be of importance for cell flexibility and gas delivery.<sup>2</sup> Membrane shape and deformability are controlled by the ATP-driven cytoskeleton. Because some capillaries in the microcirculation have a diameter of only half that of a red cell, loss of flexibility and deformability is a serious storage lesion responsible for removal of rigid cells (Fig 4-1).

The red cell membrane also contains a number of transport proteins such as the glucose transporter,  $\text{Ca}^{2+}$ -ATPase, the  $\text{Na}^{+}$ - $\text{K}^{+}$ -ATPase, the GSSG (oxidized glutathione) transport ATPases, and amino acid transporters, in addition to various other transport channels.

A prime metabolic activity of the red cell is maintaining osmotic stability through the activity of its membrane pumps, which are ATP driven. The  $\text{Na}^{+}$ - $\text{K}^{+}$ -ATPase is highly sensitive to changes in temperature and scarcely functions at 4°C. During storage in the cold, sodium diffuses into the cells and potassium leaks out until a new equilibrium is reached. The leakage of potassium is further increased by irradiation. Increased potassium content in plasma or in the additive solution of stored Red Blood Cell (RBC) units presents a potential hazard to neonates, although under most other circumstances it can be ignored.<sup>15</sup> Citrate in plasma increases potassium toxicity, while reducing red cell supernatant volume results in less potassium leakage before equilibrium is reached. Studies performed over 30 years ago indicate that restoration of potassium after transfusion is slow, and can take more than 6 days.<sup>15</sup> Surprisingly, there are no reports on restoration of potassium after transfusing RBC units with modern anticoagulant and additive solutions.

Although the macromolecules of the membrane are produced by red cell precursors, some of the components of the membrane are metabolically quite active. For example, cholesterol in the membrane exchanges readily with cholesterol in the plasma, phosphatidylinositol undergoes active phosphorylation, and some proteins are phosphorylated by protein kinases and dephosphorylated by phosphatases. These reactions may influence the functional status of membrane components, but their importance is not yet fully understood.

## Summary

In the circulation, red cells metabolize glucose by the glycolytic pathway with its pentose and 2,3-DPG shunts. The energy gained provides ATP to maintain ion and glucose concentration gradients between the plasma and erythrocyte and to secure red cell deformability. It also secures NADH to keep hemoglobin in the reduced state and NADPH to protect SH-groups on hemoglobin and membrane proteins. Finally, the production of 2,3-DPG is important for the optimal dissociation of oxygen from hemoglobin while the rise and fall of non-hemoglobin-bound 2,3-DPG with each pass through the circulatory system induces repetitive changes in the membrane-cytoskeleton architecture,

which could have implications for red cell flexibility and gas transport.

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## Red Cell Preservation in Transfusion Medicine

### General Considerations and Principles

RBCs are the most commonly transfused blood components, and their use in a variety of physical circumstances and clinical conditions has shaped their development as products. As an example, the US military's need to reduce the weight and breakage of blood bottles during shipment led to the development and adoption of plastic blood bags.<sup>22</sup> This led to closed-system component production and, fortuitously, exposure of red cells to the plasticizer diethylhexyl phthalate (DEHP), which reduced hemolysis during storage.<sup>23</sup> Arguments between blood bankers, who wanted to remove the plasma to make albumin and coagulation factors, and surgeons, who wanted to keep the plasma so the blood could flow quickly in trauma patients, led to the development of additive solutions.<sup>24</sup> Again, there were unforeseen consequences; the removal of plasma led to better RBC storage and reduced transfusion reactions. Regulatory agencies have also played a role. The US Food and Drug Administration (FDA) recognizes that 4 million patients a year receive RBCs and that the product must be safe for all.<sup>25</sup> Adding antibiotics to blood bags to prevent bacterial contamination, which kills five patients a year in the country, may seem like a beneficial advance. However, the agency must consider the accompanying risk of exposing millions of patients to allergenic and toxic drugs, with the probability of greater harm than good being achieved.

As a result of many competing forces, the development and adoption of new RBC storage technology has been slow.<sup>26</sup> Five major improvements occurred in the last 50 years: 1) the addition of phosphate, 2) the use of plastic bags, 3) the addition of adenine, 4) the development of additive solutions, and 5) the use of leukocyte reduction, which reduces hemolysis. The reasons for this slow progress have had to do with the perception of little need for change, limited investment in change, lack of understanding of the red cell storage lesion, poor developmental strategies, and conservative regulatory stances.<sup>27</sup>

For 60 years, it has been a societal goal that RBC transfusion should be available, safe, effective, and cheap.<sup>24</sup> Making red cells readily available requires 1) the ability to take liquid units out of the refrigerator and administer them immediately to critically ill or injured patients and 2) the ability to find rare units in frozen national or international inventory. Storage systems contribute to blood safety by isolating individual units in closed systems and reducing product breakdown and bacterial growth through cold storage. A major goal of storage systems is to maintain effectiveness by preserving the lifespan and function of fresh red cells to the greatest extent possible. Keeping blood components inexpensive remains a goal, but the social costs of unsafe blood are high, and the costs of additional testing have added significantly to production costs. At the present time, the costs of RBC units are

rising (see Chapter 1), and controlling those costs has been one factor in limiting progress on storage systems.

### A Short History of RBC Storage Systems

Rous and Turner developed the first red cell storage solution in 1916, a simple mixture of citrate and glucose.<sup>28</sup> It was used initially to store rabbit red cells for heterophil agglutination testing for syphilis, but when the cells appeared to be intact 4 weeks later, they were reinfused back into the donor rabbits, raising the hematocrit without increasing the reticulocyte count or bile in the urine.<sup>29</sup> A year later, Robertson used the Rous-Turner solution to build the first successful blood bank in the Harvard Medical Unit attached to the British Expeditionary Force in France.<sup>30</sup>

The major problem with the Rous-Turner solution was that it could not be heat sterilized, because the sugar caramelized; thus, there was a risk of bacterial contamination from the open mixing of the ingredients and addition of the solution to the bottles. In 1943, Loutit and Mollison solved this problem by lowering the pH of the solution to 5.0 to make acid-citrate-dextrose (ACD) solution.<sup>31</sup> ACD could be autoclaved in sterile vacuum bottles and was used as the standard blood collecting solution in the United States and Britain for many years. It was made first to be used at a 1:4 ratio with collected whole blood and later concentrated to be used at a 1:7 ratio, as is now standard, to reduce the dilution of the blood.

During research on why red cells seemed to do better in the lower volume ratio of the anticoagulant-nutrient solution, it became clear that the cells passively lose phosphate. This loss could be prevented by adding phosphate to the solution.<sup>31</sup> Citrate-phosphate-dextrose (CPD) in a 1:7 volume ratio, 63 mL for 450 mL of whole blood, preserved red cells slightly better than the older ACD.<sup>32</sup> CPD became the standard anticoagulant in the United States, but ACD persisted in Europe. Both were used for 21-day storage with recovery of 70% to 80% of the red cells 24 hours after reinfusion to their original donor.

At about the same time, Gabrio and colleagues recognized that nucleotides were important for red cell metabolism, but almost a decade passed before researchers determined that adenine was the critical intermediate.<sup>33-36</sup> In 1968, Shields formulated a mixture of CPD and adenine, CPDA-1, and showed that it markedly improved whole blood storage.<sup>37</sup> However, during the subsequent 11 years that the FDA debated the safety of adenine, plastic bags revolutionized blood banking, and this excellent whole blood storage solution turned out to work less well with the red cells left behind when plasma was removed.<sup>38</sup> Beutler and West went on to show that the higher the hematocrit of red cells in concentrates, the lower the red cell ATP concentrations and the in-vivo recovery.<sup>39</sup> The obvious answer was to add back more volume and nutrients in the form of an "additive solution," but the initial attempt to do this with a solution of bicarbonate, adenine, glucose, and phosphate (BAGP), did not work.<sup>40</sup> CPDA-1 was finally licensed as a 5-week storage solution in 1979, but only after Hogman had developed a simple additive solution of saline,

adenine, and glucose (SAG) that also worked for 5-week storage but without the adverse effects associated with the high pH.<sup>41</sup> SAG went on to immediate use in Sweden.

The use of SAG was associated with 1% hemolysis by the end of 5 weeks of storage, so mannitol was subsequently added (SAG-M) as a "membrane stabilizer."<sup>42</sup> This reduced the hemolysis by more than 50%. Drawing whole blood into CPD, making component products, and storing the concentrate with an additional 100 mL of SAG-M (CPD/SAG-M) is the standard RBC storage system in Europe. Minimal variants of this basic solution, additive solution-1 (AS-1) and additive solution-5 (AS-5), are widely used in the United States. Also used is a more substantial variant using citrate and phosphate in the place of mannitol (AS-3 or SAG-CP). All of the variants appear to be equivalent and provide about 80% recovery with about 0.3% hemolysis after 6 weeks of storage.<sup>26</sup>

The most important limits of the first generation of additive solutions are the loss of membrane with resulting loss of deformability and viability that occur with prolonged storage. The progressive loss of 2,3-DPG may also be important in some situations, although the clinical impact is not well understood. More advanced additive solutions have been demonstrated in principle in small human trials, but only one is licensed and only in a few countries.<sup>26</sup> They appear to hold the potential for 7 to 8 weeks of storage, but are considered to be attractive more for their ability to improve recovery, reduce membrane loss, and improve 2,3-DPG and/or ATP concentrations for all stored RBCs.

### Collection and Separation Procedures

The volume of whole blood removed for storage, processing, and transfusion was historically 450 mL (a pint) in Western countries. For some newer collection systems, this amount has been increased to 500 mL (a half-liter) to increase the collection with each donation and to offset the losses associated with leukocyte reduction by filtration. Products derived from either collection volumes, whether leukocyte-reduced or not, are considered one unit. The inter-donor differences in hematocrit and platelet count are sufficiently great that the yields of red cells, platelets, and plasma in components prepared from donor units of either collection volume show considerable overlap. Some have argued for a more standard definition of a unit, perhaps based on grams of hemoglobin, but it would make blood collection more difficult and wasteful.<sup>43</sup>

As noted above, the volume of anticoagulant-nutrient solution is normally 1/7th the volume of the collected blood, 63 mL for a 450-mL collection and 70 mL for a 500-mL collection. This volume ratio has been a standard for more than 50 years and was used with the anticoagulant-nutrient solutions ACD-A, CPD, and CPDA-1. There has long been a question of whether the first few drops of blood to enter the collection system are injured by their sudden immersion in the acid anticoagulant, but the red cells seem to tolerate this process with minimal hemolysis or loss of viability.<sup>44,45</sup> At the end of collection, venous blood with a pH of about 7.35 has been mixed with anticoagulant-nutrient solution with a pH of 5.0 to 5.6 with a resulting pH about 7.05 in the

mixture. The high buffer capacity of the hemoglobin molecule limits the effects of the acidic primary collection solution.

Whole blood in anticoagulant-nutrient solution was licensed for storage for 3 or 5 weeks, but has now been largely replaced by separately stored blood components. Making components from whole blood not only serves more patients but also improves the storage of the individual blood elements. Red cells are best stored cold, platelets at room temperature, and plasma, frozen. Removing the white cells also improves RBC storage by removing a cell population with high energy requirements and potential for damaging red cells by released enzymes.<sup>46</sup>

Schemes for separating whole blood into components are based on centrifugation. The standard “platelet-rich plasma” method used in the United States involves performing a low-speed “soft” spin to sediment the red cells against the pole of the bag opposite the connections to the satellite bags and then squeezing off the supernatant platelet-rich plasma from the top of the bag into the first satellite bag with pressure applied to the sides of the base of the bag. Thus, typically, 500 mL of whole blood with a hematocrit of 42%, consisting of 210 mL of red cells and 290 mL of plasma, is collected into 70 mL of anticoagulant-nutrient solution, increasing the volume in the bag to 570 mL and reducing the hematocrit to 36%. Removing most of the supernatant plasma will increase the hematocrit in the bag to 80% to 90%, consisting of the original 210 mL of red cells and the remaining 22 to 45 mL of plasma/anticoagulant-nutrient solution mixture. Of the original 290 mL of plasma and 70 mL of anticoagulant-nutrient solution, about 90% is removed in this initial separation process. In Europe an initial hard spin is generally preferred in order to harvest buffy coat for the production of platelets and increase the volume of the separated plasma. As noted above, the red cells do better if most of the plasma volume and anticoagulant-nutrient solution are replaced.

Recently, separation of whole blood into plasma and RBC by using a hollow-fiber filtration system has been described.<sup>47</sup> Red cell parameters were similar to those obtained when routine centrifugation methods were used, and the filter did not cause hemolysis. Levels of plasma Factor VIII and Factor XI were slightly reduced with this prototype; however, there was no evidence of activation of the coagulation or complement systems and filters that do not interact with coagulation factors can be made.

### Anticoagulant-Nutrient Solutions

Anticoagulant-nutrient solutions were developed from ACD to CPD to CPDA-1. This path is the result of the discovery of the critical nutrients for red cells during prolonged storage: dextrose, phosphate, and adenine. The recognition that RBC concentrates occasionally ran out of glucose led to development of CP2D, with twice the amount of glucose in CPD, and a never-licensed CPDA-2 with a third more glucose than CPDA-1.<sup>48,49</sup> The molar contents of the five licensed anticoagulant-nutrient solutions are shown in Table 4-1. Note that all of the anticoagulant-nutrient solutions are acidic, reducing the pH of the stored blood below 7.2 and leading to the rapid depletion of 2,3-DPG as it enters the glycolytic pathway.

**Table 4-1.** Compositions and Properties of Some of the Common Acid Citrate Preservative Solutions

|                                                | ACD-A | ACD-B | CPD  | CPDA-1 | CP2D |
|------------------------------------------------|-------|-------|------|--------|------|
| Citric acid (in mM/L)                          | 35    | 21    | 14   | 14     | 14   |
| Sodium citrate (in mM/L)                       | 97    | 58    | 116  | 117    | 117  |
| Dextrose (in mM/L)                             | 136   | 81    | 141  | 142    | 284  |
| Monosodium phosphate (in mM/L)                 | —     | —     | 15.8 | 16     | 16   |
| Adenine (in mM/L)                              | —     | —     | —    | 2      | —    |
| pH                                             | 5.0   | —     | 5.6  | 5.6    | 5.6  |
| Volume ratio used (anticoagulant: blood in mL) | 1:7   | 1:4   | 1:7  | 1:7    | 1:7  |

ACD = acid-citrate-dextrose (two formulations, A and B); CPD = citrate-phosphate-dextrose; CPDA-1 = citrate-phosphate-dextrose-adenine; CP2D = CPD with double dextrose.

The amounts of the nutrients in the anticoagulant-nutrient solutions are critical depending on the intended storage times. There is enough glucose in whole blood to keep the red cells healthy for 4 days. Whole blood, stored in citrate alone for up to 4 days, was considered the safest form of storage before autoclavable solutions containing sugar were developed. The high glucose content of CP2D is necessary because it is used with an additive solution that does not contain sufficient glucose, AS-3. Phosphate exists at 1 to 1.3 mM/L in plasma, but needs to be present at higher concentrations in the nutrient solution to passively diffuse against the high concentrations in the red cells when 2,3-DPG breaks down. Adenine is needed for storage only beyond 3 weeks and is an ingredient of all of the additive solutions.

In emergencies in remote locations, it may be necessary to collect fresh whole blood before new supplies of fully tested components are available. Under these circumstances, drawing whole blood into the primary collection bag containing the anticoagulant-nutrient solution and holding the whole blood at room temperature for up to 24 hours is associated with good short-term preservation of function of the blood components.<sup>50</sup> The national blood services of Finland and Israel collect all of their whole blood on one day and process it on the next, holding it at 20°C overnight in the anticoagulant-nutrient solution before separating it into components.<sup>51,52</sup>

### Additive Solutions

The first widely used additive solution, SAG, represented an attempt to replace the volume and sugar lost with plasma removal and add the adenine necessary for storage beyond 3 weeks.<sup>41</sup> It was made with normal saline with 4.5% w/v glucose and 2 mM/L of adenine. Stored RBCs had good viability for 5 weeks but 1% hemolysis. In screening a large number of compounds for additives that would reduce the hemolysis, mannitol was identified as a compound that both reduced hemolysis and had an excellent safety record with intravenous infusion. The addition of 30 mM mannitol (SAG-M) reduced

hemolysis and increased the osmolarity of the solution further.<sup>42</sup> The solutions are made acidic to a pH of about 5.6 with hydrochloric acid to allow the glucose to be heat sterilized, but the solutions have essentially no buffer capacity so they do not make the RBC units much more acidic than they were when separated from plasma. SAG-M and its close relatives, AS-1 and AS-5, are the most widely used additive solutions.

Other widely used first-generation additive solution systems include CP2D/AS-3 in the United States and Canada, and CPD/MAP (mannitol, adenine, phosphate) in Japan.<sup>53</sup> Table 4-2 provides a comparison of the ingredients and concentrations of the more common of these first-generation additive solutions.

Second-generation additive solutions started with attempts to rebalance the final suspending solution and a search for additional nutrients for the RBC units. With BAGP, Beutler attempted to preserve both ATP and 2,3-DPG by raising the pH.<sup>40</sup> However, raising the pH substantially above 7.2 led to high concentrations

of 2,3-DPG at the expense of ATP and no improvement in storage function. The composition of a representative group of second-generation additive solutions are shown in Table 4-3.

The pH of red cells during storage is very important. Above pH 7.2, the bifunctional enzyme diphosphoglycerate mutase/phosphatase converts almost all 1,3-DPG into 2,3-DPG, depriving the cell of new ATP.<sup>54</sup> Below a pH of about 6.4, the activities of the initial enzymes of glycolysis, hexokinase and phosphofructokinase, are too low to support ATP production. In this narrow pH range between 7.2 and 6.4, hemoglobin, the mineral salts in the suspension, and bicarbonate all serve to buffer the protons produced by glycolysis. The approximately 60 g of hemoglobin present in an RBC unit can buffer about 8 mEq of protons in that pH range.<sup>55</sup> However, conventional first-generation acidic additive solutions, which result in an initial pH of 7.0, fail to take advantage of a quarter of that pH range and buffer capacity. Adding 20 mM/L of phosphate delivers 2 mM in the 100 mL of additive solution and buffers about 1 mM of additional protons. Adding 20 mM/L of bicarbonate again delivers 2 mM to the final RBC suspension, which will be protonated to make carbonic acid and converted to carbon dioxide and water by red cell carbonic anhydrase. The resulting solution will buffer 2 mM of protons as the carbon dioxide diffuses out of the plastic bag. Attention to formulation and pH balance in the design of second-generation additive solutions can almost double the amount of ATP energy available to stored red cells by depressing diphosphoglycerate mutase activity while sustaining glycolysis.

Finding additional critical nutrients has been less successful. The only approved second-generation solution is phosphate, adenine, glucose, guanosine, and saline (PAGGS)-mannitol.<sup>56</sup> Guanosine was added because GTP was detected in red cells and known to decrease during storage. However, guanosine nucleotides play only a minimal role in critical events in red

**Table 4-2.** Compositions (in mM/L) of the Common Licensed First-Generation RBC Additive Solutions

|                         | SAG  | SAG-M | AS-1 | AS-3 | AS-5 |
|-------------------------|------|-------|------|------|------|
| NaCl                    | 150  | 150   | 154  | 70   | 154  |
| Phosphate               | —    | —     | —    | 23   | —    |
| Adenine                 | 1.25 | 1.25  | 2    | 2    | 2    |
| Glucose                 | 45   | 45    | 111  | 55   | 45   |
| Mannitol                | —    | 30    | 41.2 | —    | 29   |
| Citric acid             | —    | —     | —    | 2    | —    |
| Na <sub>3</sub> citrate | —    | —     | —    | 30   | —    |

SAG = saline-adenine-glucose; SAG-M = saline-adenine-glucose-mannitol; AS-1 = Adsol (Fenwal, Lake, Zurich IL), a SAG-M variant formula; AS-3 = Nutricel (Pall Medical, East Hills, NY); AS-5 = Optisol (Terumo, Somerset, NJ), a SAG-M variant formula.

**Table 4-3.** Composition and Properties (in mM/L) of Some of the Proposed Second-Generation RBC Additive Solutions

|                           | BAGP-M | PAGGS-M | PAGGG-M | ErythroSol-1 | ErythroSol-2 | EAS-81 |
|---------------------------|--------|---------|---------|--------------|--------------|--------|
| NaCl                      | —      | 72      | —       | —            | —            | —      |
| Na gluconate              | —      | —       | 72      | —            | —            | —      |
| Bicarbonate               | 115    | —       | —       | —            | —            | 26     |
| Phosphate                 | 1      | 32      | 32      | 20           | 18           | 12     |
| Adenine                   | 1      | 2       | 2       | 1.5          | 1.3          | 2      |
| Glucose                   | 55     | 52      | 52      | 45           | 38           | 80     |
| Mannitol                  | 27     | 55      | 40      | 40           | 50           | 55     |
| Guanosine                 | —      | 1.5     | 1.5     | —            | —            | —      |
| Na <sub>3</sub> citrate   | —      | —       | —       | 25           | 21           | —      |
| pH of solution            | —      | 6.3     | 6.3     | 7.4          | 8.8          | 8.4    |
| pH of red cell suspension | —      | 6.9     | 6.9     | 7.2          | 7.3          | 7.2    |
| Volume                    | 100    | 110     | 110     | 114          | 150          | 110    |

BAGP-M 5 bicarbonate-adenine-glucose-phosphate-mannitol, the original additive solution developed by Beutler; PAGGS-M 5 phosphate-adenine-glucose-guanosine-saline-mannitol, licensed in Germany; PAGGG-M 5 phosphate-adenine-glucose-guanosine-(sodium) gluconate-mannitol, developed by de Korte as a chloride-free variant of PAGGS-M; ErythroSol-1 and -2 developed by Hogman; EAS-81 5 developed by Hess and Greenwalt.

cell storage (inhibiting the primitive coagulation enzyme transglutaminase) and the solution worked only modestly better than first-generation additive solutions with a 74.6% 24-hour in-vivo recovery after 7 weeks in the only published series.

As ingredients were added to advanced additive solutions, the salt concentration was generally reduced to maintain osmotic balance. Reducing the chloride concentration in the suspending solutions caused intracellular chloride to passively leave the red cells, but this can occur only if other anions countered the flow, and phosphate, bicarbonate, and hydroxyl ions are the only available anions.<sup>57</sup> The influx of these anions initially increases the intracellular pH, but eventually the pH falls. PAGGG-M, in which the sodium chloride is replaced by sodium gluconate, is an example of an attempt to take advantage of the “chloride shift” phenomenon to maintain red cell 2,3-DPG.<sup>58</sup>

The ErythroSol solutions were developed by Hogman based on the ideas of using the “chloride shift” to increase the intracellular pH while using a more alkaline final suspending solution and additional phosphate to simultaneously maintain ATP.<sup>59</sup> ErythroSol-1 used half-strength citrate (0.5 CPD) and had problems with incomplete anticoagulation in blood collected from donors with low (but acceptable) hematocrit levels. ErythroSol-2 uses full strength CPD and an alkaline additive solution made with disodium phosphate. In both systems the glucose was placed in a separate bag from the rest of the additive solution during manufacture and sterilization and added only at the time of RBC component production. The original description of ErythroSol-2 suggested that the optimal pH had been determined to preserve both ATP and 2,3-DPG.<sup>60</sup> However, the study needs confirmation because only 10 units were stored in the ErythroSol-2. The natural variability in donor hematocrit means that some units with low red cell content have low buffer capacity and, therefore, become overly alkaline. The solution can achieve successful 7-week storage. Merely increasing the pH of conventional additive solutions to increase the 2,3-DPG causes the ATP concentrations to fall.<sup>61</sup>

The experimental additive solution-81 (EAS-81) was the end result of work by Hess and Greenwalt that reexamined the use of bicarbonate as a buffer to prevent erythro-apoptosis by maintaining high concentrations of red cell ATP and limited hemolysis with mannitol and hypotonic conditions.<sup>54</sup> Formulating the solutions to get the starting pH as close as possible to 7.2 preserves the 2,3-DPG concentrations for 2 weeks.<sup>62</sup> The buffering provided by the bicarbonate probably allows RBC storage for at least 8 weeks based on six recovery measurements.<sup>62</sup>

Finally, increasing the volume of these advanced additive solutions increases the buffering capacity and allows greater storage time. With 300 mL of additive solutions, Hess and colleagues were able to achieve storage times of 10 to 12 weeks, but the higher volume and lower storage hematocrit probably make such solutions inappropriate for clinical use, especially in infants and massively transfused trauma patients.<sup>63</sup>

### **Additional Factors Influencing RBC Quality** **Temperature and Time Lapses during Collection and Component Preparation**

Whole blood is collected at body temperature and must be maintained at room temperature if platelets are to be prepared from the collection. By present US regulations, whole blood must be separated into its components in 8 hours if platelets and Fresh Frozen Plasma are to be manufactured. If only RBCs and frozen plasma are to be made, the whole blood can be held on ice for up to 24 hours. The problems with holding blood at room temperature are twofold. Red cells metabolize glucose at higher rates when warm, producing more lactate and protons that reduce the pH and further slow metabolism. In addition, labile coagulation factors are lost. Attempts to validate the 24-hour warm hold for 6-week RBC storage using first-generation additive solutions have not been successful. However, in Israel and Finland, where this system is used, RBCs are rarely stored for that long because the national blood services are efficient and supply lines are short.

As noted above, fresh whole blood is occasionally collected in emergencies on the battlefield or in isolated locations such as Pacific Island nations and stored at room temperature. Based on the above experience and experimental data, blood probably maintains reasonable functionality for 24 to 72 hours if maintained between 19 and 25°C.<sup>52</sup> However, such use can be recommended only in the most urgent of situations.

### **Temperature and Time Lapses during Storage**

RBCs that have been allowed to rewarm to greater than 10°C during storage are considered unfit for transfusion and are destroyed per FDA regulations. This has led to the wide use of the “30-minute rule,” meaning that units are discarded if they have been off ice for more than 30 minutes. Experimental work has shown that glucose is metabolized about 10 times as fast at 25°C as at 4°C and that RBCs stored at 25°C lose viability 10 times as fast. Thus, a day of storage at room temperature would reduce the in-vivo recovery equivalent to 10 days of 4°C storage.<sup>64,65</sup> The regulation is very conservative with regard to temperature but it is also intended to prevent bacterial overgrowth in contaminated units.

### **Storage Containers**

Polyvinyl chloride bags plasticized with DEHP are the standard RBC storage containers. The presence of the DEHP reduces hemolysis by 4-fold during storage by intercalating into the red cell membrane.<sup>66</sup> Other plasticizers, such as butyryl-n-trihexyl citrate, work almost as well, but are more expensive (~5% more for a multibag set with leukocyte filter) and have an unusual smell when initially unwrapped.

While questions about the safety of DEHP have been raised, they are based on very limited animal testing and must be balanced against the obvious safety value of being able to visually inspect the contents of blood bags and reduce RBC losses by extending shelf-life. The general trend in Europe to avoid the slowly degradable plasticizer, DEHP, has resulted in the



introduction of butyryl-n-trihexyl citrate plasticized multibag systems in Sweden, Spain, and Norway.

Leukocyte Reduction

Leukocyte reduction improves RBC storage by removing a highly metabolically active blood component that would make the bag more acidic sooner. White cells also secrete cytokines, and when they die after exposure to the cold, they release proteolytic, glycolytic, and lipolytic enzymes that damage the red cell surface. On the other hand, some red cells are damaged in the filters, and typically 15 to 35 mL of blood is lost in the filters, depending on filter size and whether whole blood or concentrated red cells are being leukocyte reduced. Red cells from donors with sickle cell trait and mild thalassemia may also clog filters. Leukocyte reduction improves the red cell 24-hour in-vivo recovery by several percent according to the best estimates.<sup>46</sup>

Washing

Washing RBCs in saline to remove the plasma causes small losses of red cells in the bag transfers, as well as loss of the supporting nutrients, glucose, phosphate, and adenine. Because the cells are used soon after washing, generally within 6 to 24 hours, only the glucose loss is physiologically important. There is still a small amount of glucose inside the washed red cells, but it is metabolized quickly especially if the cells are not promptly refrigerated. In the absence of glucose, metabolism stops and the cells are very susceptible to oxidative stress and erythro-apoptotic changes.

Irradiation

During storage at 4°C, red cells lose potassium. This potassium collects in the supernatant fluid in the closed storage bag at a rate of about 1 mEq/day until equilibrium is reached between intra- and extracellular concentrations, usually at about 60 to 70 mEq/L depending on the storage hematocrit (Table 4-4). Gamma irradiation in doses of 2500 cGy, given to prevent graft-vs-host disease, damages the red cell membranes and increases this rate of potassium loss to approximately 1.5 mEq/day; however the reinfused red cells have normal in-vivo survival.<sup>67</sup> The current FDA regulation that irradiated RBCs expire 28 days after irradiation limits the potential maximum potassium concentration by limiting the period of potassium loss. Nevertheless, care must be taken in all situations when large volumes of older, high-potassium RBC units are used to prime cardiopulmonary bypass, dialysis, or apheresis circuits and then administered at high flow rates into the central circulation.

Pathogen Reduction

Pathogen reduction systems developed so far require additional manipulation of and stresses on the stored red cells that are system specific. Examples from two such proposed systems are illustrative. Diethanolamine (DEA) was proposed as a red cell permeable nucleic acid cross-linker with broad pathogen killing potential and minimal direct red cell toxicity. However,

**Table 4-4.** Effect of Storage Duration on Characteristics of RBC Concentrates in AS-1

|                      | 35 Days<br>(n = 25) | 42 Days<br>(n = 10) | 49 Days<br>(n = 10) | 56 Days<br>(n = 10) |
|----------------------|---------------------|---------------------|---------------------|---------------------|
| % recovery           | 86                  | 82                  | 76                  | 71                  |
| % hemolysis          | 0.28                | 0.32                | 0.51                | 0.68                |
| ATP $\mu$ M/g Hb     | 3.1                 | 2.7                 | 2.3                 | 2.3                 |
| [K+] meq/L in plasma | 45                  | 50                  | 52                  | 60                  |

Unpublished data obtained from the US Food and Drug Administration under the Freedom of Information Act. ATP = adenosine triphosphate; Hb = hemoglobin.

the system required exposure of the cells to DEA for 20 hours at room temperature and extensive secondary washing to reduce the remaining amounts of this carcinogenic chemical. When the time- and temperature-related decrease in red cell pH was added to the effects of washing with large volumes of acidic solutions and storage in an acidic additive solution, red cell recovery suffered.<sup>68</sup> Riboflavin, a photoactive oxidizer, has also been proposed as a highly red cell permeable molecule for pathogen reduction in conjunction with ultraviolet light. However, red cells are so optically dense that the units must be diluted and transferred to large bags only a few millimeters thick for photo-treatment, and the photo-treatment must be done above a critical temperature. The bag transfers all involve losses of red cells, the choice of diluent fluid is important, and the hemoglobin and other optically active molecules in the red cells are also damaged by the light exposure. The red cells need additional energy to attempt to correct the damage. Finally, the reconstituted and treated red cells need to be stored in an additive solution that is balanced to maximize their life span.

Functionality

Since the discovery of the effect of 2,3-DPG on the oxygen affinity of hemoglobin, it has been common to discuss the functionality of stored red cells in terms of their 2,3-DPG content.<sup>69</sup> This is an oversimplification for several reasons. First, while it is clear that low 2,3-DPG can affect oxygen delivery in animal models at critically low hemoglobin concentrations, these concentrations are well below standard transfusion triggers.<sup>70</sup> Second, 2,3-DPG works in red cells by binding and stabilizing deoxyhemoglobin. This moves the base of the oxygen-hemoglobin dissociation curve to the right and increases the P50, but most oxygen transport occurs at the top of the binding curve which is relatively unaffected, and even in critical situations, the arterial PO<sub>2</sub> is higher than the P50. However, 2,3-DPG may play an important role in red cell membrane transport and cytoskeleton architecture because of its interaction with attachment points between the cell membrane and the cytoskeleton, and the interaction of deoxyhemoglobin with band 3. These interactions, the critical importance of oxygen transport, and the retrospective data that

suggest an association between adverse patient outcomes and increased storage time of banked RBCs, drives continuing efforts to reduce 2,3-DPG depletion during storage with modern additive solutions. Until that is achieved, it is recommended in some countries, such as Norway, to use RBCs in SAG-M, not older than 10 days, for the transfusion of intensive care patients.

Probably more important for normal red cell function is the loss of membrane, cell deformability, and ATP secretion that occurs with storage. Direct observation of the flow characteristics of fresh, stored, and stored rejuvenated RBCs suggests that ATP secretion in response to decreased shear, by which red cells dilate small vessels to maintain their forward flow, is most important.<sup>71</sup> Membrane deformability, which is regulated by the ATP-modulated cytoskeleton, is very important for the passage of red cells through capillaries half their diameter. Maintaining high ATP concentrations is, therefore, an important function of modern additive solutions.

### Rejuvenation

Red cell loss of viability during storage is different from red cell senescence in the body. In the body, red cells undergo cumulative oxidative damage that leads to reduced enzyme activities and cross-linking of cytoskeletal components. These are essentially irreversible processes and are ultimately associated with macrophage clearance of old red cells, possibly mediated by phosphatidyl serine exposure or neoantigen formation. The changes that lead to loss of viability during storage are largely reversible by a process called “rejuvenation.” Hogman showed that rejuvenating red cells at the end of 6 weeks of storage in SAG-M increased their 24-hour in-vivo recovery from 77% to 89%.<sup>72</sup>

This rejuvenation is a strictly metabolic recharging of red cells at the end of their storage period. Such cells have a low pH as well as low ATP and 2,3-DPG concentrations. They can be rejuvenated by incubation in a high pH solution of phosphate, inosine, pyruvate, and adenine (PIPA, Rejuvesol, Cytosol Laboratories, Braintree, MA) for 2 hours. Such incubation increases their ATP and 2,3-DPG concentrations and increases their in-vivo recovery, probably by allowing them to internalize negatively charged membrane phospholipids that would otherwise signal clearance by macrophages. Return of the normal distribution of phospholipids also prevents red cells from participating in plasma coagulation reactions. Rejuvenation does not reverse the oxidative damage to band 3 of the cell membrane. However, the fact that RBC can be rejuvenated at the end of storage by increasing their pH and ATP suggests that improved storage could be achieved by a method designed to maintain pH and ATP. This is the fundamental idea behind second-generation additive solutions.

### Frozen Storage of RBCs

RBCs can be frozen, and in the frozen state they are stable for long periods—Valeri has reported storage of 37 years.<sup>73</sup> Four methods of freezing RBCs have been extensively tested, two have been developed for practical use, and one remains in common use.<sup>74</sup>

During red cell freezing, water turns to ice and the salt concentration of the remaining intracellular water increases, drawing in more water and expanding the cell. Under normal circumstances, this leads to cell rupture. Rupture can be prevented by freezing the cells so rapidly that water does not have time to enter, or by diluting the total cellular water with a cryoprotectant so that not enough water enters to rupture the cell. While several cryoprotectants can work, glycerol is the standard material used because of its cost and safety. The two systems for RBC cryopreservation that have been developed for clinical use both use glycerol. One system uses a “low” glycerol concentration of about 20% and rapid cooling by plunge freezing in liquid nitrogen; the other uses a “high” glycerol concentration of about 40% and slow cooling in  $-80^{\circ}\text{C}$  freezers. The low glycerol frozen RBCs must be maintained in the vapor phase of liquid nitrogen whereas the high glycerol frozen RBCs are stable at temperatures below  $-65^{\circ}\text{C}$ . The high cost of maintaining liquid nitrogen freezers and the difficulty of transporting products frozen in liquid nitrogen limit the utility of the low glycerol system. High glycerol frozen RBCs can be transported on dry ice. When the RBCs are thawed, the glycerol must be removed promptly to prevent it from poisoning the red cell metabolism and to protect the recipient because the glycerol-loaded red cells would swell and rupture if placed directly into the bloodstream. Thus, once thawed, the RBCs must be deglycerolized by washing in a set of graded salt solutions. In the past, the glycerolization and deglycerolization of RBCs for freezing were open manual processes, so the thawed RBCs had to be used within 24 hours or discarded.

The recent partial automation of a closed system for glycerolizing and deglycerolizing RBCs now allows them to be kept after thawing in the liquid state for 2 weeks.<sup>75</sup> RBCs collected in any of the standard licensed systems can be frozen up to 6 days after collection and stored for up to 10 years. The deglycerolized RBCs are then stored in AS-3. Net losses of red cells in the freeze-thaw-wash process are approximately 5% to 15%, and the 24-hour in-vivo recovery of infused red cells is about 78% after 2 weeks. Frozen RBCs entail substantial costs for processing and storage, probably four times that of a standard liquid unit, so only for the rarest blood units is there an advantage for frozen inventory.

Other systems of freezing and freeze-drying have been demonstrated, but are associated with greater than 1% hemolysis and so would require a washing step before administration. This limitation prevents their use in emergency medicine, and they are too costly and labor intensive to compete with glycerol frozen RBCs.<sup>74</sup>

### Validation of RBC Storage Systems

RBC storage systems have historically been validated by demonstrating that the stored cells do not hemolyze during storage and that they circulate normally after reinfusion. Measures of normal circulation have included increments in recipient hemoglobin, and determining the recovery and in-vivo half life of the transfused red cells. Transfused red cells have been counted using differential agglutination, radioactive tracer labeling, and flow-cytometric differential counting.

Chromium-51 labeling has been the standard method used in the United States for 50 years. There is a published standard method, but only a handful of laboratories perform the measurement, and a recent attempt to gather a decade's experience identified only 900 measurements.<sup>76</sup> Chromium-51 labeling persists in the United States because it is the only validated method compatible with autologous red cell reinfusion, and the infectious disease risks of allogeneic RBCs are considered unacceptable for storage system development work. The methods of red cell labeling have been the subject of an excellent review.<sup>77</sup>

When performing chromium-51 labeling, it is important to recognize that red cells from different donors may have very different 24-hour in-vivo recovery values. In a typical study of a modern additive solution with an 84% mean recovery, individual RBC units have recoveries as high as 95% and as low as 65%.<sup>48</sup> These differences in viability correlate with differences in the red cell ATP concentrations, but poorly ( $r^2 = 0.4$ ).

There have been attempts to measure the functionality of red cells, as opposed to their survival. Presumably, the functionality of red cells is related to their ability to deliver oxygen to tissues and to flow in the microcirculation. However, none of the red cell rigidity or microcirculatory flow measures is widely available or validated. The level of 2,3-DPG is just a surrogate marker for red cell function and is limited by the difficulty of performing the test. In a recent shared sample exercise, 12 of the world's premier laboratories could not consistently measure 2,3-DPG.<sup>78</sup>

Finally, in countries that do not perform chromium-51 recovery measures, there has been a trend to use the ATP level as a red cell quality measure. While the ATP is a poor surrogate for recovery in small clinical trials, its central role in the inhibition of erythro-apoptosis probably gives it special importance. Unfortunately the measure is not very reproducible from laboratory to laboratory, so a wide margin of safety is required.<sup>78</sup>

## Summary

RBC storage systems work remarkably well, making red cells for transfusion available, safe, effective, and cheap for the populations who can organize effective national blood systems. They are a product of slow and empiric development, and our present systems do not reflect our current understanding. They can be expected to improve in the future.

## Acknowledgment

The authors would like to thank Stein Holme, PhD, for valuable discussions and comments on this chapter.

## Disclaimer

The authors have disclosed no conflicts of interest.

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# 5

## Red Cell Immunology and Compatibility Testing

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Immunohematology is the study of immune aspects of blood. This chapter begins with a review of the basic concepts of immunity as they apply to the formation of antibodies to foreign antigens, also called *allo*antibodies. Other areas of immunohematology such as autoantibodies and drug-induced antibodies are presented elsewhere.

The relevance of immunohematology in transfusion medicine is addressed through detailed discussion of the principles of serologic tests and their application to donor-recipient compatibility testing. Identification of the blood group specificity of alloantibodies (alloantibody identification) encountered during such testing is presented in a step-by-step manner such that a limited number of possible specificities remain. Additional testing may be required to obtain a definitive answer. The chapter concludes with discussion of computer-based programs to prevent release of ABO-incompatible units from the blood bank, as well as new technologies, including nanotechnology, that prevent transfusion errors caused by patient misidentification.

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### Red Cell Immunology

#### The Immune Response

Immune responses can be divided into two categories: humoral immunity and cell-mediated events. Humoral immunity is B-cell mediated and entails the generation of antibodies. Cell-mediated immunity is a reflection of the action of T cells, which regulate the immune response through the production of cytokines and direct interactions with other elements/cells of the immune system.

An individual's genetic constitution determines the characteristics of that person's cells and fluid-phase molecules; this is

referred to as the "self" of the individual. All other ("non-self") molecules are considered foreign. During fetal development, and continuing through the neonatal period, B and T cells produce receptors that enable each lymphocyte to recognize a single specific antigen.<sup>1</sup>

#### Antigen Receptors of T Cells and B Cells

The antigen receptors of both T and B cells consist of two polypeptide chains synthesized under the direction of two different chromosomal loci. The loci for B-cell receptors are on chromosome 14 and either on chromosome 2 or 22; the loci for T-cell receptors are on chromosome 7 and 14 (but not the same locus as for B-cell receptors). In the early stages of lymphocyte development, the genetic material at each of these loci undergoes a process of rearrangement that is unique to each cell.<sup>2</sup>

Germline DNA at these loci contain numerous base-pair sequences, each capable of encoding different amino acid sequences. The gene rearrangement process entails selection of single base-pair sequences for each portion of the immunoglobulin molecule from the assortment (library) of base pairs available (Fig 5-1), and also involves elimination of unused genetic material.<sup>3</sup>

Each T-cell/B-cell receptor chain consists of a constant portion and a variable portion. Amino acid sequences of the variable portion determine the idiotypic specificity of the immunoglobulin molecule. For both receptors, one chain contains selections from three libraries (V, D, J), while the other chain contains selections from V and J libraries. Given that the heavy chain locus has as many as 400 exons in the V library, 20 in the J library, and six in the D library, the number of possible combinations for different protein configurations is astronomic. At the light chain loci there are some 300 possible selections in the V library, and four in the J library. Selection from each library is random, and further amino acid sequence variation is introduced by inaccurate splicing or when different exons are joined together. Consequently,  $10^9$  different receptor configurations can be generated.<sup>1</sup>

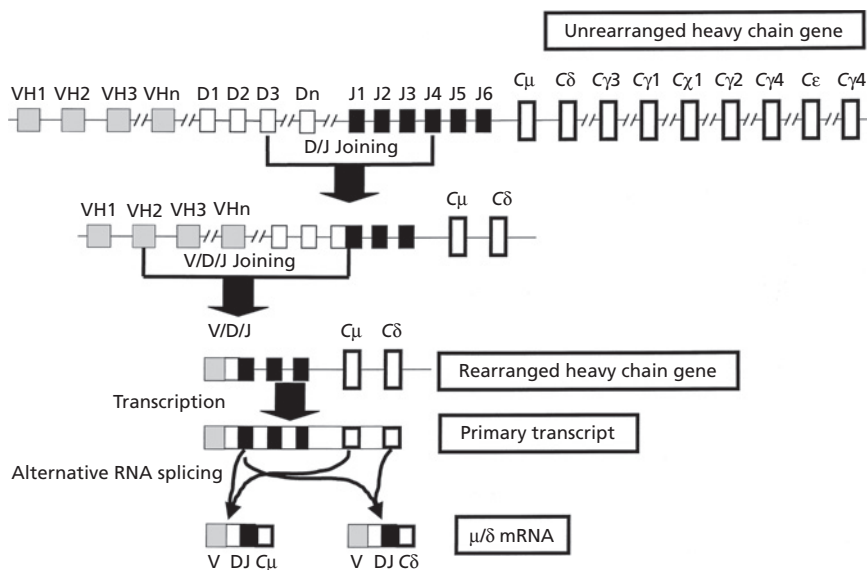


**Figure 5-1.** Diagram of the immunoglobulin heavy chain locus on chromosome 14. In the unrearranged locus (top) V, D, and J libraries are on the left; sequences for the constant regions are on the right. Gene rearrangement occurs by selection of one D and one J exon to form a D/J unit. This is then joined by one V exon. The V/D/J unit is adjacent to the exons for  $\mu$  and  $\delta$  constant regions. Through alternative splicing, the primary transcript generates either  $\mu$  or  $\delta$  heavy chains with the same variable region configuration (idiotypic). In later generations, clonal progeny can attach the V/D/J transcript to other sequences from the constant region (isotype switch).

### Exposure to Foreign Protein Antigens

If foreign protein antigens enter the host, antigen-presenting cells (APCs) isolate the antigen and display the molecular configurations as epitopes on their cell surfaces. T and B lymphocytes with the appropriate receptor for the unique molecular configuration can then establish intimate contact with the APC. This contact initiates cellular events that generate clones of activated lymphocytes specific for the foreign antigen. Cytokines produced by APCs and by activated T cells cause B cells to evolve into immunoglobulin-secreting plasma cells. In turn, B cells capture antigen and present it back to T cells in a manner that causes the T cells to secrete cytokines that promote more focused antibody production.

Most plasma cells have a short but active life of antibody production. Some, however, become memory cells that retain intracellular changes resulting from exposure to APCs; these persist in the circulation long after their initial activation.

### Primary Response

The first immunoglobulin class to appear in the bloodstream is always IgM; the lag phase (time between sensitizing event and appearance of antibody) can vary from days to months. Shortly after the appearance of IgM antibody, IgG antibody of the same specificity usually becomes detectable. The IgM component disappears over time, while the IgG component persists indefinitely. Cytokines from activated T cells are essential for this isotype switching and for generation of memory cells.

### T-Cell Independent Response

It is of note that antibodies to carbohydrate antigens are invariably IgM. This is because B-cell APCs can interact directly with complex polysaccharides such as those that carry blood group A or B activity. Structures that have multiple identical repeat carbohydrate chains can initiate B-cell proliferation and antibody secretion without the action of T cells. In the absence of the

immunomodulatory effects of T cells, isotype switching does not occur, and no memory cells develop.<sup>4</sup>

### Secondary (Anamnestic) Response

Within a few hours or days after subsequent exposure to T-dependent antigen, there is a sharp rise in the level of IgG antibody. Because there has been prior clonal expansion, the number of participating B cells will be high, resulting in production of significantly (eg, 100-fold) more IgG than was produced in the primary response. Cytokines secreted by T cells promote more focused antibody production.

### Affinity Maturation

The affinity of IgG antibody for specific antigen increases progressively during the secondary immune response, particularly after stimulation with low doses of antigen. This affinity maturation is most pronounced after secondary challenge with antigen.

One explanation for affinity maturation is clonal selection. A second explanation for affinity maturation is that, after a class switch has occurred in the immune response, somatic mutations occur that fine-tune the antibodies to be of higher affinity. There is experimental evidence for this mechanism, although it is not known how the somatic mutation mechanism is activated after exposure to antigen.

### Clonality

Only antigen-specific B cells proliferate and mature into antibody-secreting plasma cells. The number of progenitor B cells that are initially activated will determine the heterogeneity (clonality) of the immune response. The response will range from polyclonal (when many different B cells are activated simultaneously) to monoclonal (when the antibody-producing cells originate from a single ancestral B cell). Monoclonal antibodies for reagent use can also be produced in vitro using hybridoma technology.<sup>5</sup>

## Immunoglobulin Molecules

Figure 5-2 portrays the basic structure of an antibody molecule, which consists of four polypeptide chains: two identical light chains of 214 amino acids, and two identical heavy chains of 440 or more amino acids. Antigenic specificity is conferred by the 134 N-terminal amino acids of the light chains and the 144 N-terminal amino acids of the heavy chains; these are described as variable regions. The remaining portions of both chains are referred to as constant regions.<sup>6</sup>

### Light Chains

The constant region of the light chains can have one of two different amino acid sequences, designated kappa ( $\kappa$ ) and lambda ( $\lambda$ ).  $\kappa$  chains are encoded by a locus on chromosome 2,  $\lambda$  chains by a locus on chromosome 22. A single cell will synthesize an immunoglobulin molecule containing either  $\kappa$  or  $\lambda$  chains, but never both.

### Heavy Chains

The constant region of heavy chains can manifest one of five amino acid sequences, designated by the Greek letters  $\alpha$  (alpha),  $\delta$  (delta),  $\epsilon$  (epsilon),  $\gamma$  (gamma), and  $\mu$  (mu). These give rise to

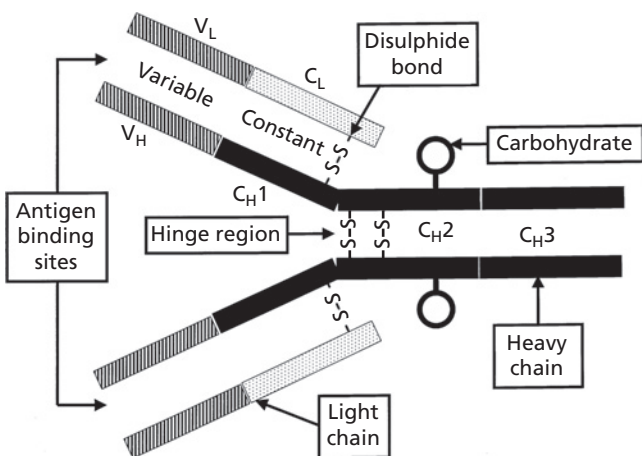


**Figure 5-2.** Basic structure of an immunoglobulin molecule.

five types of immunoglobulin classes termed IgA, IgD, IgE, IgG, and IgM, respectively. The site on chromosome 14 that governs the manufacture of heavy chains contains DNA sequences for all five types of heavy chain.

### Antibody Diversity

Serologic studies have revealed a number of antigenic markers on the heavy and light chains of immunoglobulin molecules. The results of such studies are strongly supported by molecular analysis of the immunoglobulin genes.<sup>3,6</sup> Differences between these antigenic markers are illustrated in Fig 5-3.

### Isotypes

Isotypes result from structural changes to the constant region heavy and light chains. Genes encoding the various isotypes (IgA, IgD, IgE, IgG, IgM,  $\kappa$ , and  $\lambda$ ) are all present in the human genome. Antibodies for isotype determination can be prepared by heterologous immunization (eg, mouse immunization with human serum).

### Allotypes

Allotypic distinctions between antibodies are the result of genetic polymorphisms, and each marker is not present in every individual. This genetic variation results from different alleles at structural loci. Antibodies to allotypic variants can be raised by same-species (homologous, allogeneic) immunization, and are often found in the sera of women who have given birth to infants with different allotypic markers. These markers usually represent amino acid changes in the constant regions of antibody molecules.

### Idiotypes

Idiotypic variation is the result of differences in amino acid sequence of the variable regions of either light or heavy chains. Antibodies to idiotypes may recognize sequences either within or outside the antigen-combining site, and can be prepared by either homologous or heterologous immunization.

### Blood Group Antibodies

Blood group antibodies are immunoglobulins that react with antigens on the surface of red cells. They can either be acquired

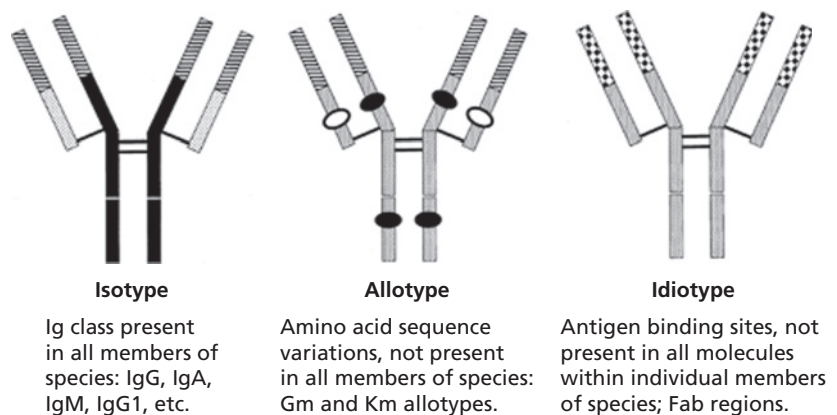


**Figure 5-3.** Diversity of antibody molecules.



Table 5-1. Characteristics of Blood Group Active Immunoglobulins

| Immunoglobulin Characteristic | IgM             | IgG         | IgA                  |
|-------------------------------|-----------------|-------------|----------------------|
| H-chain isotype               | μ               | γ           | α                    |
| Subclasses                    | 2               | 4           | 1                    |
| L-chain types                 | κλ              | κλ          | κλ                   |
| Sedimentation constant        | 19 S            | 7 S         | 11 S                 |
| Molecular weight              | 900-1000 kD     | 150 kD      | 180-500 kD           |
| Electrophoretic mobility      | Between β and γ | γ           | γ                    |
| Serum concentration (mg/dL)   | 85-205          | 1000-1500   | 200-350              |
| Antigen binding sites         | 10 (5)          | 2           | 4                    |
| Fixes complement              | Often           | Some        | No                   |
| Placental transfer            | No              | Yes         | No                   |
| Direct agglutinin             | Yes             | Usually not | Usually not          |
| Hemolytic in vitro            | Often           | No          | No                   |
| Example                       | Anti-A, anti-B  | Anti-D      | Anti-Lu <sup>a</sup> |

naturally or through immunization with foreign red cells.<sup>7</sup> Most blood group antibodies are either IgG or IgM; occasionally they may be IgA. The IgD and IgE immunoglobulins have not been implicated as blood group antibodies,<sup>8</sup> and will not be discussed further.

Physical Properties

The physical properties and serologic characteristics of the three immunoglobulin classes involved with blood group specificity are summarized in Table 5-1. IgM antibodies have 10 available antigen binding sites, have a wide spanning distance, and often fix complement to red cells. Antibodies to A and B antigens are predominantly IgM; they are found normally in persons whose red cells lack the corresponding antigen. They are stimulated by antigens present in the environment. Bacteria constituting normal intestinal flora carry blood group A- and B-like polysaccharides. As these flora establish themselves in the gut, they provide the immune stimulus for anti-A and anti-B.<sup>7</sup> Anti-A and anti-B formed in this manner are often referred as “natural” antibodies. They are also called “expected” antibodies, because in adults with a normal immune system these antibodies are almost always present when the corresponding antigens are absent on the red cells.

In contrast to anti-A and anti-B, most other blood group antibodies are IgG, which are immune in origin and do not appear in plasma/serum unless the host is exposed directly to foreign red cell antigens. The stimulating event is usually blood transfusion or pregnancy. IgG antibodies are smaller than IgM, and have only two sites for antigen binding.

All antibodies to red cell antigens, other than naturally occurring anti-A and anti-B, are considered unexpected. They can be either alloantibodies, directed toward non-ABO-system antigens absent on the red cells of the antibody producer, or autoantibodies, directed toward self-antigens. The latter may cause autoimmune hemolytic anemia. Unexpected antibodies in donor plasma may destroy recipient red cells, while antibodies in the

recipient may cause accelerated destruction of transfused red cells. In pregnant women, such antibodies may cross the placenta and cause hemolytic disease of the fetus and newborn.<sup>8</sup>

Close to 300 different blood group alloantibodies have been described.<sup>9</sup> Each antibody reacts with its specific antigen on the surface of red cells. The immunoglobulin class and clinical relevance of some of the antibodies encountered during compatibility testing are shown in Table 5-2.

Red Cell Antigen-Antibody Interactions

Red cells normally repel one another. The force of repulsion (zeta potential) that exists at the red cell surface depends not only on the electronegative surface charge, but also on the ionic cloud that normally surrounds it.<sup>10,11</sup> The electronegative charge is imparted primarily by the carboxyl (COO<sup>-</sup>) group of N-acetyl-neuraminic acid,<sup>12</sup> which is a constituent of alkali-labile tetrasaccharides that are attached to MN and Ss sialoglycoproteins (glycophorins A and B, respectively).<sup>9</sup> At a minimum, there are 3.2 × 10<sup>7</sup> of these charged groups per red cell. The magnitude of this charge is modified by the formation of an ionic cloud of positively charged (+) sodium ions and negatively charged (−) chloride ions that align in alternating sequence extending from the red cell membrane surface (Fig 5-4). The net effect of this ionic cloud is to decrease the electrostatic repulsion between red cells.

In considering the interaction of red cell antigens with blood group antibodies, three variables must be considered: the surface charge of the red cells, the dielectric constant of the medium (a relative measure of its charge dissipation), and the mutual attraction between antigen and antibody. The latter involves electrostatic (coulombic) and hydrogen bonds, and van der Waals interactions, which hold antigen and antibody together.<sup>10</sup> Further, there must be structural complementarity between antigen and its binding site on antibody molecules.<sup>13</sup>

There are two phases of red cell antigen-antibody interactions; these often occur simultaneously. The first phase is one of association, involving binding of antibody to antigens on the red cell membrane. The second phase involves the formation of an agglutination lattice of antibody-coated cells. For the latter to occur, antibody molecules must be able to span the distance between adjacent red cells.

Interaction between red cells and blood group antibodies can be observed either directly by examination of red cell and antibody mixtures for agglutination (or clumping) and/or hemolysis, or indirectly by use of the antiglobulin test. These two serologic techniques are summarized in Table 5-3.

Direct Agglutination

IgM antibody molecules can span the distance that exists between red cells when suspended in saline, and cause direct agglutination of those cells if they carry the corresponding antigen. This direct agglutination can be observed when red cells are mixed with IgM antibody, briefly incubated at room temperature, and centrifuged. Examination for hemagglutination may be performed either macroscopically or microscopically, but the latter is not

**Table 5-2.** Characteristics of Blood Group Alloantibodies

| Antibody                         | Agglutinating | Coating* | Ig Class <sup>†</sup> | C3-binding | Effect of Ficin | HDFN <sup>‡</sup> | HTR <sup>§</sup> | % Compatible | Comments                                    |
|----------------------------------|---------------|----------|-----------------------|------------|-----------------|-------------------|------------------|--------------|---------------------------------------------|
| A                                | ✓             | ✓        | M, G                  | ✓          | ↑               | ✓                 | ✓                | 56           | No dosage                                   |
| A <sub>1</sub>                   | ✓             | Some     | M                     | ✗          | ↑               | ✗                 | Rare             | 64           | In 2% A <sub>2</sub> , 25% A <sub>2</sub> B |
| At <sup>a</sup>                  |               | ✓        | G                     | ✗          |                 | ✗                 | ✓                | Rare         | In Blacks                                   |
| B                                | ✓             | ✓        | M, G                  | ✓          | ↑               | ✓                 | ✓                | 85           | No dosage                                   |
| Bg                               |               | ✓        | G                     | ✗          |                 | ✗                 | ✗                |              | To HLA <sup>  </sup>                        |
| C                                | Some          | ✓        | G, M                  | ✗          | ↑               | ✓                 | ✓                | 30           |                                             |
| c                                | Some          | ✓        | G, M                  | ✗          | ↑               | ✓                 | ✓                | 0            | Often with anti-E                           |
| Ch                               |               | ✓        | G                     | ✗          | ↓               | ✗                 | ✗                | 2            | HTLA <sup>¶</sup> ; to C4d <sup>#</sup>     |
| C <sup>w</sup>                   | Some          | ✓        | G, M                  | ✗          | ↑               | ✓                 | ✓                | 98           |                                             |
| Co <sup>a</sup>                  |               | ✓        | G                     | ✗          |                 | ✓                 | ✓                | Rare         |                                             |
| Co <sup>b</sup>                  |               | ✓        | G                     | Rare       |                 | ✓                 | ✓                | 91           |                                             |
| Cr <sup>a</sup>                  |               | ✓        | G                     | ✗          |                 | ✗                 | ✓                | Rare         | In Blacks                                   |
| Cs <sup>a</sup>                  |               | ✓        | G                     | ✗          |                 | ✗                 | ✗                | 2            | HTLA                                        |
| D                                | Some          | ✓        | G, M                  | ✗          | ↑               | ✓                 | ✓                | 15           | No dosage                                   |
| Di <sup>a</sup>                  | Some          | ✓        | G                     | ✗          |                 | ✓                 | ✓                | >99          | **                                          |
| Di <sup>b</sup>                  |               | ✓        | G                     | ✗          |                 | ✓                 | ✓                | Rare         |                                             |
| Do <sup>a</sup>                  |               | ✓        | G                     | ✓          | ↑               | ✗                 | ✓                | 33           |                                             |
| Do <sup>b</sup>                  |               | ✓        | G                     | ✓          | ↑               | ✗                 | ✓                | 13           |                                             |
| E                                | Some          | ✓        | G, M                  | ✗          | ↑               | ✓                 | ✓                | 70           | Often with anti-c                           |
| e                                | Some          | ✓        | G, M                  | ✗          | ↑               | Rare              | ✓                | 3            | Auto in WAIHA <sup>††</sup>                 |
| f(ce)                            | Some          | ✓        | G, M                  | ✗          | ↑               | ✓                 | ✓                | 36           | Compatible with c–                          |
| Fy <sup>a</sup>                  | Rare          | ✓        | G                     | Rare       | ↓               | ✓                 | ✓                | 33           |                                             |
| Fy <sup>b</sup>                  |               | ✓        | G                     | Rare       | ↓               | ✓                 | ✓                | 20           |                                             |
| Ge                               | Some          | ✓        | G, M                  | ✓          | ↓Some           | ✗                 | ✓                | Rare         |                                             |
| H                                | ✓             | ✓        | M, G                  | ✓          | ↑               | ✓                 | ✓                | Rare         | In O <sub>n</sub> blood                     |
| Hy                               |               | ✓        | G                     | ✗          |                 | ✗                 | ✓                | Rare         | In Blacks                                   |
| I                                | ✓             | Some     | M, G                  | ✓          | ↑               | ✗                 |                  | Rare         | In i adults                                 |
| Jk <sup>a</sup>                  | Some          | ✓        | G                     | ✓          | ↑               | ✓                 | ✓                | 25           |                                             |
| Jk <sup>b</sup>                  | Some          | ✓        | G                     | ✓          | ↑               | ✓                 | ✓                | 25           |                                             |
| JMH                              |               | ✓        | G                     | ✗          | ↓               | ✗                 | ✗                | Rare         | HTLA                                        |
| Js <sup>a</sup>                  |               | ✓        | G                     | ✗          |                 | ✓                 | ✓                | >99          |                                             |
| Js <sup>b</sup>                  |               | ✓        | G                     | ✗          |                 | ✓                 | ✓                | Rare         | In Blacks                                   |
| K                                | Some          | ✓        | G, M                  | Rare       |                 | ✓                 | ✓                | 90           |                                             |
| k                                |               | ✓        | G                     | ✗          |                 | ✓                 | ✓                | Rare         |                                             |
| Kn <sup>a</sup>                  |               | ✓        | G                     | ✗          | ↓               | ✗                 | ✗                | 2            | HTLA                                        |
| Kp <sup>a</sup>                  |               | ✓        | G                     | ✗          |                 | ✓                 | ✓                | 98           |                                             |
| Kp <sup>b</sup>                  |               | ✓        | G                     | ✗          |                 | ✓                 | ✓                | Rare         |                                             |
| Lan                              |               | ✓        | G                     | Some       |                 | ✓                 | ✓                | Rare         |                                             |
| Le <sup>a</sup>                  | ✓             |          | M                     | ✓          | ↑               | ✗                 | Rare             | 78           |                                             |
| Le <sup>bl</sup>                 | ✓             |          | M                     | ✓          | ↑               | ✗                 | ✗                | 28           | True anti-Le <sup>b</sup>                   |
| Le <sup>bH</sup>                 | ✓             |          | M                     | ✓          | ↑               | ✗                 | ✗                | 48           | In A <sub>1</sub> and A <sub>1</sub> B      |
| Lu <sup>a</sup>                  | ✓             | Some     | G, A                  | Rare       |                 | Rare              | ✗                | 92           |                                             |
| Lu <sup>b</sup>                  |               | ✓        | G, A                  | Rare       |                 | ✓                 | ✓                | Rare         |                                             |
| M                                | ✓             | Some     | M, G                  | Rare       | ↓               | Rare              | Rare             | 22           |                                             |
| McC <sup>a</sup>                 |               | ✓        | G                     | ✗          | ↓               | ✗                 | ✗                | 1.5          | HTLA                                        |
| N                                | ✓             | Some     | M, G                  | ✗          | ↓               | Rare              | Rare             | 28           | Potent in N–U–                              |
| P <sub>1</sub>                   | ✓             | Some     | M                     | Some       | ↑               | ✗                 | Rare             | 21           | No dosage                                   |
| P+P <sub>1</sub> +P <sup>k</sup> | ✓             | ✓        | M, G                  | ✓          | ↑               | ✓                 | ✓                | Rare         |                                             |
| Rg                               |               | ✓        | G                     | ✗          | ↓               | ✗                 | ✗                | 3            | HTLA; to C4d                                |
| S                                | Rare          | ✓        | G                     | Some       | ↓               | ✓                 | ✓                | 45           |                                             |
| s                                | Rare          | ✓        | G                     | Rare       | ↓               | ✓                 | ✓                | 11           |                                             |
| Sc1                              |               | ✓        | G                     | ✓          |                 | ✗                 | ✗                | Rare         |                                             |
| Sc2                              |               | ✓        | G                     | ✗          |                 | ✓                 | ✗                | 99           |                                             |

(continued)

Table 5-2. (continued)

| Antibody        | Agglutinating | Coating* | Ig Class <sup>†</sup> | C3-binding | Effect of Ficin | HDFN <sup>‡</sup> | HTR <sup>§</sup> | % Compatible | Comments  |
|-----------------|---------------|----------|-----------------------|------------|-----------------|-------------------|------------------|--------------|-----------|
| SJ <sup>a</sup> |               | ✓        | G                     | ✗          | ↓               | ✗                 | ✗                | 2            | HTLA      |
| U               |               | ✓        | G                     | ✗          |                 | ✓                 | ✓                | Rare         | In Blacks |
| V               | Some          | ✓        | G                     | ✗          | ↑               | ✓                 | ✓                | >99          |           |
| Vel             | Some          | ✓        | M, G                  | ✓          | ↑               | ✗                 | ✓                | Rare         |           |
| Wr <sup>a</sup> | Some          | ✓        | M, G                  | ✗          |                 | ✓                 | ✓                | >99          |           |
| Xg <sup>a</sup> |               | ✓        | G                     | ✗          | ↓               | ✗                 | ✗                | 23           |           |
| Yk <sup>a</sup> |               | ✓        | G                     | ✗          | ↓               | ✗                 | ✗                | 8            | HTLA      |
| Yt <sup>a</sup> |               | ✓        | G                     | ✗          | ↓ Some          | ✗                 | Some             | Rare         |           |
| Yt <sup>b</sup> |               | ✓        | G                     | ✗          |                 | ✗                 | ✗                | 92           |           |

\* Coating antibodies detected primarily through the use of the indirect antiglobulin test.  
† Predominant immunoglobulin class shown first.  
‡ HDFN = reported to cause hemolytic disease of the fetus and newborn.  
§ HTR = reported to cause hemolytic transfusion reactions.  
|| HLA = antibody to antigen present on white blood cells that is variably expressed on red cells.  
¶ HTLA = antibody, usually of high titer, to antigen of low site density on red cells. Incorrectly called high-titer, low avidity antibody.  
# C4d = antibody to epitopes found on the fourth component of human complement (C4).  
\*\* Rare in Whites and Blacks. Seen almost exclusively in individuals of Mongolian ancestry (South American Indians, Chippewa Indians, Chinese, Japanese).  
†† WAIHA = seen as an autoantibody in some cases of warm autoimmune hemolytic anemia.

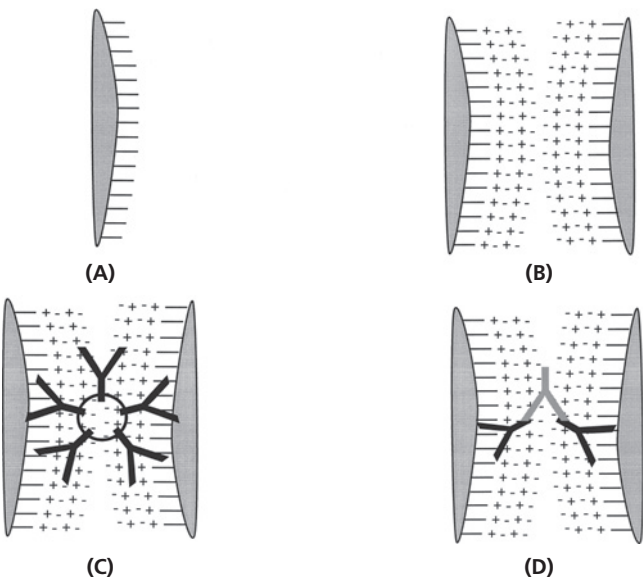


**Figure 5-4.** (A) Red cells carry a strong negative electric charge, imparted primarily by the carboxyl (COO<sup>-</sup>) group of N-acetyl-neuraminic acid. (B) When suspended in a saline medium, red cells are kept apart by virtue of their negative surface charge. The magnitude of this charge is modified by the formation of an ionic cloud of positively charged (+) sodium ions and negatively charged (-) chloride ions that align in alternating sequence extending from red cell membrane surface. The force of repulsion that exists between red cells in suspension is referred as the zeta potential. (C) In order for antibody molecules to cause agglutination of adjacent red cells, the antigen binding sites must be able to span the intercellular distance. Pentameric IgM antibody molecules can readily bridge the distance between adjacent red cells. (D) IgG molecules (black) cannot readily effect direct agglutination. However, they are able to coat red cells. This antibody coating can be detected by a second IgG antibody (eg, rabbit antihuman IgG), depicted here in gray.

Table 5-3. Two Types of Red Cell Serologic Tests

| Direct Tests                             | Indirect Tests                                       |
|------------------------------------------|------------------------------------------------------|
| Mix serum and red cells                  | Mix serum and red cells*                             |
| Incubate (room temperature) <sup>†</sup> | Incubate (37°C)                                      |
| Centrifuge                               | Centrifuge                                           |
| Examine for agglutination and hemolysis  | Examine for agglutination and hemolysis <sup>‡</sup> |
|                                          | Wash, to remove unbound globulins                    |
|                                          | Add antihuman globulin reagent                       |
|                                          | Centrifuge                                           |
|                                          | Examine for agglutination <sup>‡</sup>               |

\* An enhancement reagent, to promote antibody uptake, may be incorporated here.  
† This step is optional.  
‡ Confirm negative test results with IgG-coated red cells.

encouraged because it leads to the detection of nonspecific reactivity. The strength of the observed reactions can be graded, and numerical values can be assigned based on a scoring system in common use.<sup>14</sup>

**Hemolysis**

IgM antibodies, in particular anti-A and anti-B, can also cause direct lysis of antigen-positive red cells, especially if tests are incubated at 37°C. This hemolysis results from the action of complement, a series of α and β globulins that act in sequence as enzymes (eg, esterases) to form a complex (membrane attack complex) that causes holes to be formed in the red cell membrane through which the intracellular hemoglobin can escape (see Fig 5-5). For initiation of the complement cascade, the “tails” (Fc portions) of two

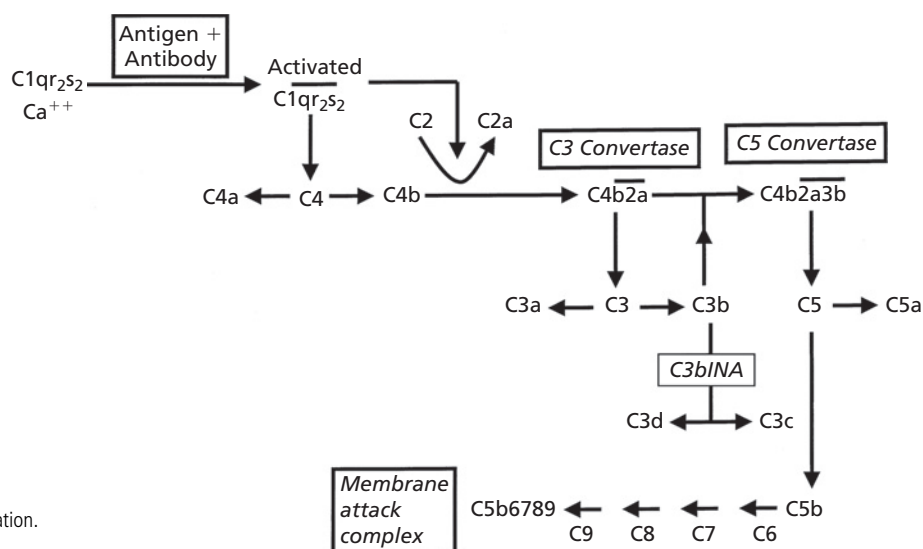


**Figure 5-5.** Classical pathway for complement activation.

pairs of immunoglobulin heavy chains must be in close proximity to each other on the red cell surface. Initiation of the complement cascade readily occurs when a single pentameric IgM antibody molecule is bound; for it to occur with IgG antibodies there must be closely adjacent heavy chain regions of two IgG molecules at the cell surface. Further, not all IgM antibodies bind complement to red cells and complement binding does not always proceed to complete red cell lysis by membrane attack complex (intravascular hemolysis). Rather, activated C3b may be cleaved to C3d, which does not initiate the binding of C5; hence, no membrane attack complex is formed. However, C3d remains bound to red cells and can be detected by the antiglobulin test (see below).<sup>8,14</sup>

Red cells coated with C3b are also removed by cells of the reticuloendothelial system (extravascular hemolysis). However, there are no receptors on macrophages for C3d. Thus, inactivators of C3b serve to limit the degree of both intravascular and extravascular hemolysis.

### Antiglobulin Test

IgG antibodies do not readily cause direct agglutination of red cells, because intercellular distances are wider than the spanning distance of the antigen-binding sites of the IgG molecule. However, some IgG antibodies will cause direct agglutination if the red cell zeta potential is reduced. This can be accomplished by treating the cells with proteolytic enzymes such as papain or ficin, which remove the proteins that carry the negatively charged neuraminic acid residues. Alternatively, zeta potential can be decreased by raising the dielectric constant of the red cell suspending medium by the addition of colloids such as bovine serum albumin.<sup>8</sup>

IgG antibodies, as well as complement components bound to red cells by either IgM or IgG antibodies, are best detected by the antiglobulin test. This test, once called the Coombs' test, entails the use of antibodies raised in animals (usually rabbit or sheep), or prepared by hybridoma technology,<sup>5</sup> to detect human IgG and

complement bound to red cells. The principles of this test are as follows:

1. Antibody molecules and complement components are globulins.
2. The injection of human globulins, either purified or in whole serum, into an animal stimulates the animal to produce antibodies against the foreign globulins. These antibodies are referred to as antihuman globulins (AHGs). The antihuman globulins that are important for blood group serologic work are anti-IgG and anticomplement (anti-C3d).
3. Antihuman globulin will react with human globulins, either bound to red cells or free in serum. Thus, red cells must be washed free of unbound globulins before testing with AHG. This is crucial to the avoidance of false-negative tests caused by neutralization of AHG by unbound globulins.
4. Washed red cells coated with human globulin are agglutinated by AHG.

Antiglobulin tests can be performed either indirectly following in-vitro incubation of red cells with serum, or directly to demonstrate that red cells are coated with globulins in vivo. An indirect antiglobulin test (IAT) is used to detect and identify unexpected antibodies in the serum of blood donors, prospective transfusion recipients, and prenatal patients. The direct antiglobulin test (DAT) is used to detect antibodies bound to red cells in vivo; such antibodies may be seen in patients with hemolysis caused by autoantibodies or drugs, infants with hemolytic disease of the fetus and newborn, and patients manifesting an alloimmune response to a recent transfusion.<sup>14</sup>

### Compatibility Testing

Pretransfusion compatibility testing comprises a series of policies and procedures, including laboratory tests, the goals of which are to provide blood for transfusion that will have the

**Table 5-4.** Required Elements of Pretransfusion Testing<sup>16</sup>

| Element                                                      | Requirement                                                                                                                                                                                                                 | AABB Standards |
|--------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------|
| <b>Patient Sample Collection</b>                             |                                                                                                                                                                                                                             |                |
| <i>Identification</i>                                        | Positive identification of intended recipient and blood sample at time of collection                                                                                                                                        | 5.11           |
|                                                              | Mechanism to identify phlebotomist                                                                                                                                                                                          | 5.11.2.2       |
| <i>Label</i>                                                 | Two independent identifiers (eg, first and last names, patient unique number)                                                                                                                                               | 5.11.2         |
|                                                              | Date of collection                                                                                                                                                                                                          | 5.11.2         |
|                                                              | Attached to tube before leaving side of intended recipient                                                                                                                                                                  | 5.11.2.1       |
| <i>Timing</i>                                                | Within 3 days of red cell transfusion if patient was transfused or pregnant within previous 3 months, or if patient history is uncertain or unavailable                                                                     | 5.13.3.2       |
| <b>Donor Unit Confirmatory Testing</b>                       |                                                                                                                                                                                                                             |                |
| <i>First-time donors or if electronic crossmatch is used</i> | From an attached segment, after the ABO and Rh label has been affixed                                                                                                                                                       | 5.9.5, 5.12    |
|                                                              | Anti-A and anti-B on units labeled group A or group B                                                                                                                                                                       |                |
|                                                              | Anti-A,B on units labeled group O                                                                                                                                                                                           |                |
|                                                              | Direct tests with anti-D on units labeled Rh-negative                                                                                                                                                                       |                |
| <i>Repeat donors</i>                                         | A computer system (validated to prevent release of ABO and Rh mislabeled units) may be used                                                                                                                                 | 5.9.5.1        |
| <i>Unexpected antibodies</i>                                 | Confirmatory testing not required                                                                                                                                                                                           |                |
| <b>Patient Sample Testing</b>                                |                                                                                                                                                                                                                             |                |
| <i>ABO</i>                                                   | Red cells with anti-A and anti-B; serum or plasma with A <sub>1</sub> and B red cells; concordance between red cell and serum results                                                                                       | 5.13.1         |
| <i>Rh</i>                                                    | Test with anti-D; test for weak D not required                                                                                                                                                                              | 5.13.2         |
| <i>Unexpected antibodies</i>                                 | By a method that will demonstrate clinically significant antibodies; 37°C incubation preceding an antiglobulin test (alternative methods of documented equivalency acceptable); using reagent red cells that are not pooled | 5.13.3         |
|                                                              | Confirm negative tests with IgG-coated red cells, or control system appropriate to the method                                                                                                                               | 5.13.3.4       |
| <b>Donor/Patient Testing</b>                                 |                                                                                                                                                                                                                             |                |
| <i>Crossmatch</i>                                            | Antiglobulin test; applies if clinically significant antibodies detected, currently or in past                                                                                                                              | 5.15.1         |
|                                                              | Serologic tests for ABO incompatibility if no clinically significant antibodies detected, currently or in the past                                                                                                          | 5.15.1.1       |
|                                                              | Computer system validated on site to prevent release of ABO-incompatible blood may be used to detect to ABO incompatibility                                                                                                 | 5.15.2.1       |

optimal clinical effect without causing undue harm to the recipient. These elements of pretransfusion testing can be placed into one of three categories: those related to donor unit testing and processing, those related to patient sample collection and testing, and those that serve as a final check between the donor unit and intended recipient (Table 5-4). Proper performance of each element will enable the right unit of blood to be transfused to the right patient.<sup>15</sup>

## Donor Testing

### Collection Facility

The initial ABO, Rh, and antibody detection tests on donor blood, tests for infectious diseases, the interpretation of these tests, and correct labeling of donor units are functions normally carried out by a regional donor center. However, some hospital-based transfusion services continue to procure a portion of their blood needs from the population at large (allogeneic donors), and some collect blood from patients for their own use in

elective surgical procedures (autologous donors). In addition, some patients request that they receive blood from relatives or friends (directed donations). Regardless of the type of donor, each unit of blood must be subjected to tests for ABO, Rh, and unexpected antibodies, as well as for markers of infectious disease (see Chapter 62).

The volume of testing that needs to be performed at donor centers often necessitates use of automated equipment. Instrumentation currently in use is often based on microplate technology, and test results may be interpreted electronically. ABO grouping entails testing both donor red cells and serum/plasma. Red cells are tested with anti-A and anti-B; and the serum/plasma is tested with A<sub>1</sub> and B red cells. Although not required, anti-A, B, and A<sub>2</sub> red cells may also be used, because results with these reagents serve to detect subgroups of A that may be nonreactive in direct tests with anti-A alone.

Rh typing is performed with anti-D. Because D is highly immunogenic,<sup>8</sup> it is presumed that even weak expression of the

antigen will evoke an immune response; consequently, donor red cells that initially type as Rh-negative in direct agglutination tests are tested further for weak D, usually by an IAT (see Table 5-3). Only those units that are negative with anti-D by this method or an equivalent procedure can be labeled Rh-negative.<sup>16(p37)</sup> All straightforward D-positive and weak D-positive blood is considered Rh-positive.

### Transfusing Facility

The ABO group of all units of Whole Blood or Red Blood Cells, and the Rh group of those labeled Rh-negative, must be confirmed by the transfusion facility before transfusion.<sup>16(p43)</sup> Only tests with reagent antisera and donor red cells need to be performed; units labeled group O can be tested with anti-A,B alone.<sup>17</sup> For D typing of units labeled Rh-negative, only direct tests with anti-D are necessary; testing for weak expression of D is not required, nor is repeat testing for unexpected antibodies or for markers of infectious diseases.<sup>16(p43)</sup> For transfusion facilities that also collect blood, confirmatory testing for first-time donors must be performed after the original ABO and Rh label has been affixed using a sample from a segment attached to the donor unit. For repeat donors, the ABO/Rh may be confirmed by an electronic record check of previous donations, provided that tests for ABO compatibility are to be performed serologically. However, if blood is to be released using an electronic cross-match process (see later), the ABO/Rh of the donor unit must be confirmed serologically by testing an attached segment, even for repeat donors.

### Patient Testing

#### Sample Collection

Because the major cause of fatal, hemolytic transfusion reactions is ABO-incompatible transfusion resulting from patient/sample misidentification, the importance of the following measures cannot be overemphasized.

1. *Requisition.* Forms requesting blood and blood components must contain the first and last names and a unique numerical identifier of the intended recipient, such as a hospital registration number.<sup>16(p41)</sup> The name of the requesting physician, gender and date of birth of the patient, clinical diagnosis, and previous transfusion or pregnancy history are additional useful pieces of information.

2. *Patient Identity.* The collection of a properly labeled blood sample for pretransfusion testing from the correct patient is critical to safe blood transfusion. The person collecting the sample must positively identify the patient.<sup>14</sup> This is facilitated through use of a wristband, containing the patient's full name and unique hospital registration number that remains attached to the patient throughout the hospitalization. The information on the requisition form should be compared with that on the wristband; blood samples should not be collected if there is a discrepancy.

In the absence of a wristband, the nursing staff should follow hospital procedure for identifying and attaching a wristband to the patient before any samples are drawn. In an emergency,

a temporary identification number should be used, and cross-referenced with the patient's name and hospital identification number once they are known.

3. *Labeling.* Blood samples must be drawn into correctly labeled tubes. The tubes must be clearly labeled at the bedside with the patient's first and last names, the patient's unique hospital identification number, and the date of collection.<sup>11</sup> There must be a means of identifying the person who collected the sample. Conventionally, the phlebotomist signs or initials the tube and signs or initials the requisition form so that there is a permanent record of the phlebotomist's name.<sup>14</sup>

4. *Confirmation of Sample Identity.* Upon receipt of blood samples for pretransfusion testing, the information on the label must be compared with that on the requisition. A new sample must be obtained whenever there are discrepancies or if there is any doubt about the identity of the sample. It is unacceptable to correct an incorrectly labeled sample.

5. *Type of Sample.* Either serum or plasma may be used for pretransfusion testing, but most workers performing tube testing use serum to avoid introducing small fibrin clots into serologic tests. Such clots may be mistaken for agglutination. Fibrin clots may also form when samples from heparinized patients are collected into non-anticoagulated tubes. These samples will clot properly following the addition of protamine sulfate.<sup>14</sup> However, anticoagulated (plasma) samples are required if testing is automated.

Few workers prefer to use serum rather than plasma for compatibility testing, to facilitate detection of antibodies that primarily coat red cells with the C3d component of complement. Bound C3d will not activate the lytic phase of the complement cascade but can be detected with AHG reagents containing anti-C3d.<sup>8,18</sup> EDTA, citrate and other commonly used anticoagulants chelate calcium ions that are essential for complement activation. However, as discussed later, the use of AHG reagents containing anti-C3d for compatibility testing is not mandatory.<sup>14</sup>

6. *Age of Specimen.* To ensure that the specimen used for compatibility testing is representative of a patient's current immune status, serologic studies must be performed using blood collected no more than 3 days in advance of the transfusion when the patient has been transfused or pregnant within the preceding 3 months, or when such information is uncertain or unavailable.<sup>16(p44)</sup> From a practical standpoint, it is simpler to stipulate that all pretransfusion samples must be collected within 3 days before red cell transfusions, rather than ascertain whether or not each patient has been recently transfused or pregnant.

7. *Storage.* Blood samples used for compatibility testing, including donor red cells, must be kept at 1-6°C for at least 1 week after each transfusion.<sup>16(p42)</sup> This ensures that appropriate samples are available for investigational purposes should adverse responses to transfusion occur.

### ABO Typing

Both reagent antisera and red cells for ABO typing are available commercially. Anti-A and anti-B are monoclonal antibodies prepared by hybridoma technology. Reagent red cells are

**Table 5-5.** The Expected Reactions of the Four Common ABO Phenotypes: Results of Blood Typing Tests

| Blood Type | Anti-A | Anti-B | A <sub>1</sub> Red Cells | B Red Cells |
|------------|--------|--------|--------------------------|-------------|
| O          | 0      | 0      | 4+                       | 4+          |
| A          | 4+     | 0      | 0                        | 4+          |
| B          | 0      | 4+     | 4+                       | 0           |
| AB         | 4+     | 4+     | 0                        | 0           |

O = no agglutination; 4+ = strong agglutination

usually suspended in a preservative medium containing EDTA, to prevent lysis of the red cells by complement-binding anti-A and anti-B.<sup>14</sup> ABO grouping is performed using a direct agglutination technique (Table 5-3).<sup>19</sup> Red cells are tested with anti-A and anti-B, and the serum or plasma with known A<sub>1</sub> and B red cells. Use of anti-A,B and A<sub>2</sub> red cells is optional, but generally considered unnecessary when ABO typing potential transfusion recipients.<sup>17</sup>

The expected findings for each of the four common ABO phenotype are shown in Table 5-5. When interpreting the results of ABO grouping tests, it is important to note the reciprocal relationship that exists between the absence of A and/or B antigens on red cells and the presence of the expected anti-A and/or anti-B in the serum. If there is conflict between cell and serum ABO tests, group O blood should be provided for transfusion until the discrepancy is resolved and reliable conclusions of the patient's ABO type can be made.<sup>14</sup>

**Rh Typing**

Only tests for D are performed routinely on the red cells of prospective transfusion candidates. There are two different types of reagent anti-D available for Rh typing. High-protein reagents are prepared with human IgG anti-D diluted in bovine albumin and other substances that potentiate agglutination. Their final protein concentration may be greater than 20 g/dL. Such a high protein level is needed to potentiate IgG antibody reactivity so that positive and negative tests can be recognized almost instantaneously using a direct agglutination technique. In the United States, low-protein reagents (protein content ≤ 7 g/dL) are a blend of monoclonal IgM and monoclonal/polyclonal human IgG anti-D. With blended anti-D reagent the IgM component causes direct agglutination of Rh-positive red cells and the IgG component permits detection of the weak expression of D by application of the antiglobulin test.<sup>14</sup>

Only direct tests with blended anti-D reagents are required on patient samples; the test for weak D is not necessary.<sup>16(p43)</sup> To avoid incorrect designation of an Rh-negative recipient as Rh-positive because of autoantibodies or abnormal serum proteins, a control system appropriate to the anti-D reagent in use is required.<sup>14</sup> For low-protein anti-D, a concurrent negative test with anti-A and/or anti-B is considered an appropriate control system. Apparent AB Rh-positive samples should be retested concurrently with anti-D and an inert Rh control reagent.<sup>19</sup>

**Table 5-6.** Acceptable Methods for Pretransfusion Antibody Detection

|                                      | Serum     | Red Cells    | Incubation      | AHG*   |
|--------------------------------------|-----------|--------------|-----------------|--------|
| Saline                               | 2-3 drops | 1 drop, 3-5% | 30-60 min; 37°C | IgG/PS |
| Albumin                              | 2-3 drops | 1 drop, 3-5% | 15-30 min; 37°C | IgG/PS |
| Low-ionic-strength saline (solution) | 2 drops*  | 2 drops, 2%* | 10-15 min; 37°  | IgG/PS |
| Gel                                  | 25 µL     | 50 µL, 0.8%  | 15 min; 37°C    | IgG    |
| Polyethylene glycol                  | 2 drops†  | 1 drop       | 15-30 min; 37°C | IgG    |
| Solid-phase adherence                | 1 drop    | ‡            | 10-15 min; 37°C | IgG    |
| Low-ionic polycation                 | 100 µL    | 1 drop, 1%   | 1 min; RT       | None   |

\* Drop volumes should be equal.  
† Plus 4 volumes of 20% polyethylene glycol.  
‡ Predetermined by reagent supplier.  
AHG = antihuman globulin; PS = polyspecific AHG; RT = room temperature.

**Tests for Unexpected Antibodies**

Methods for detecting unexpected antibodies in the serum or plasma of prospective transfusion recipients must be those that detect clinically significant antibodies.<sup>16 (p44)</sup> An IAT after 37°C incubation of patient's serum or plasma with reagent red cells that are not pooled is usually required. Table 5-2 lists many of the blood group alloantibodies that can be detected during pretransfusion testing, and provides the approximate percentage of compatible units that are likely to be encountered in a predominantly Caucasian blood donor population, except as otherwise noted.

A number of options exist regarding the selection of methods for pretransfusion antibody detection (see Tables 5-6 and 5-7). Decisions relative to these options are within the purview of the blood bank medical director. They should be made based on the type of patients served, the causes and frequency of previous significant antibody-mediated transfusion reactions, the availability of resources, and with the realization that no one method will detect all clinically significant antibodies.

**Tube Test Methods**

A variety of red cell suspending media or additives are used either to enhance antibody uptake or to potentiate the agglutination phase of antibody-antigen interactions (Table 5-7). Low-ionic-strength saline (LISS) solution,<sup>20</sup> normal saline, or red cell preservatives (modified Alsever's solution) are used as red cell suspending media. Bovine serum albumin (22% or 30% w/v), LISS-additives, or polyethylene glycol (PEG) are commonly added directly to serum-red-cell mixtures.<sup>19,21</sup>

Antibody uptake (the first stage of an antigen-antibody interaction) is accelerated when red cells are suspended in LISS or PEG. The magnitude of the ionic cloud (see Fig 5-4) that forms around negatively charged red cells suspended in LISS (approximately 0.03 M) is lower than that surrounding red cells suspended in normal saline (0.15 M NaCl). This cloud not only decreases intercellular distances by reducing the zeta potential imparted by negatively charged carboxyl groups, but also hinders

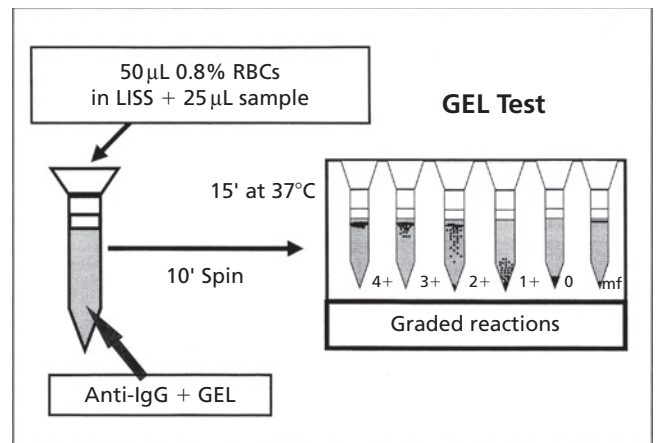
**Table 5-7.** Changes in Pretransfusion Testing Practices, 2001-2004<sup>30,31</sup>

| Test            | Specific Methods      | Percentage of US Laboratories Performing Test |      |
|-----------------|-----------------------|-----------------------------------------------|------|
|                 |                       | 2001                                          | 2004 |
| ABO/Rh          | Tube                  | 97.4                                          | 91.6 |
|                 | Gel                   | 1.4                                           | 7.7  |
|                 | Liquid microplate     | 1.1                                           | 0.6  |
| Antibody screen | Gel                   | 27.5                                          | 41.1 |
|                 | LISS-tube             | 48.2                                          | 40.2 |
|                 | PEG-tube              | 8.6                                           | 7.7  |
|                 | Albumin-tube          | 8.7                                           | 5.9  |
|                 | Saline-tube           | 3.2                                           | 2.2  |
|                 | Solid-phase adherence | 3.3                                           | 2.4  |
|                 | Liquid microplate     | 0.5                                           | 0.5  |
|                 | Immediate-spin        | 42.4                                          | 39.6 |
| Crossmatch      | Electronic (computer) |                                               | 2.0  |
|                 | Gel                   | 17.9                                          | 29.3 |
|                 | LISS-tube-IAT         | 26.5                                          | 19.8 |
|                 | PEG-tube-IAT          | 3.6                                           | 3.1  |
|                 | Albumin-tube-IAT      | 6.6                                           | 4.3  |
|                 | Saline-tube-IAT       | 2.6                                           | 1.6  |
|                 | Solid-phase adherence | 0.2                                           | 0.2  |
|                 | Liquid microplate     |                                               | 0.1  |

association between antibody and antigen; thus, reducing the magnitude of the ionic cloud promotes antibody association.<sup>10</sup> Weakening of the ionic cloud also causes an increase in attraction between the negatively charged red cell membrane and positive charges on the antigen-binding sites of antibody molecules. In addition, PEG competes for and removes water molecules at the red cell surface. Similar to the ionic cloud of  $\text{Na}^+$  and  $\text{Cl}^-$  ions that forms around red cells suspended in saline, these water molecules also sterically hinder antibody association. The net effect of these phenomena is that more antibody can be bound in a shorter period of time using LISS or PEG as opposed to saline. Consequently, incubation times can be reduced to 10 to 15 minutes for LISS, or 15 to 30 minutes for PEG, compared to 30 to 60 minutes for saline.<sup>22</sup>

In contrast, albumin promotes the second stage of antigen-antibody interactions, namely the agglutination of antibody-coated red cells. It does so by increasing the dielectric constant of the suspending medium, thereby reducing the zeta potential (force of repulsion) between red cells.<sup>11</sup> Because of this effect, albumin can promote the direct agglutination of IgG-coated red cells (see Judd<sup>19</sup> for a method). However, it should be noted that albumin is not used in the United States in a manner that promotes direct agglutination of antibody-coated red cells. Rather, the enhancing effect of albumin on red cell antigen interactions can be attributed to its formulation as a low-ionic solution.

In the low-ionic polycation technique, serum is incubated with red cells suspended in LISS to enhance antibody uptake.<sup>23</sup> The second phase of the reaction is facilitated by aggregating red

**Figure 5-6.** The gel test for detecting unexpected antibodies.

cells with the polycation, Polybrene. Aggregation is reversed with sodium citrate, but agglutinates formed by antigen-antibody interactions are not dispersed.

It is common practice to examine saline, albumin, and LISS tests for direct agglutination before subjecting them to an IAT. These examinations can be made immediately after mixing cells and serum together (immediate-spin tests) and again after incubation at 37°C. For antiglobulin testing, either anti-IgG or polyspecific AHG (containing anti-IgG and anti-C3) may be used.<sup>14</sup> However, use of polyspecific AHG leads to the detection of an inordinate number of unwanted positive tests,<sup>24</sup> and its use in routine antibody detection tests is not recommended.<sup>17</sup> Because of nonspecific aggregation and uptake of complement components that can occur in PEG tests, the tests should not be examined for direct agglutination and should not be tested with antiglobulin reagents containing anti-C3.<sup>21</sup>

### Column Technologies

A gel test for detecting red cell antigen-antibody interactions was first described in 1990 by Lapierre and colleagues.<sup>25</sup> Cards consisting of six microcolumns, each containing agarose gel suspended in anti-IgG, are commercially available. Atop each card is an incubation chamber in which reagent red cells and test plasma are dispensed. The cards are incubated at 37°C, then centrifuged. As the red cells pass through the gel, they are separated from the serum/plasma and come into contact with anti-IgG. If the red cells become coated with antibody during incubation, they will be agglutinated by anti-IgG. The agglutinated red cells become trapped in the gel; unagglutinated red cells pellet to the bottom of the microcolumn. A procedure for the gel test is depicted in Fig 5-6.

The gel test has proven to be equivalent to standard tube technologies (LISS; PEG) for the detection of unexpected antibodies. In one study,<sup>26</sup> the sensitivity and specificity of gel for potentially significant antibodies was 92% to 97%, respectively. This compares to 98% (sensitivity) and 90% (specificity) for a tube LISS procedure.



Column technologies offer several advantages over conventional test tube procedures:

1. Simplified testing, as follows:
  - a. Dispense measured volumes of test plasma/serum and red cells (in LISS) into gel card incubation chambers.
  - b. Transfer cards to an incubator (15 minutes) and then to a centrifuge (10 minutes).
  - c. Read and record results.
2. When compared to conventional tube tests, there is no centrifugation/reading for direct agglutination after incubation; no addition of antiglobulin reagent; no need to validate negative tests using IgG-coated red cells. Omission of these manipulations (especially the associated, repetitive transfer of test tubes between racks and centrifuges) provides significant “hands-on” time savings.
3. Increased time savings through batch testing; a technologist can perform 36 to 48 antibody screens in about the same amount of time it takes to process 12 samples by conventional tube techniques.<sup>26</sup>
4. Increased reproducibility of results, through use of measured volumes of reactants, elimination of the washing process, less subjective reading of tests.
5. Stability of reactions, facilitating validation of results by a second technologist. With conventional tube methods, there is only one opportunity to read reactions; with the gel test, reactions can be read as many times as needed up to 48 hours after centrifugation.
6. Reduction in the detection of cold agglutinins (anti-I/HI -M, -P<sub>1</sub>, -Le) of no or doubtful clinical significance with consequential reduction in the number of samples requiring antibody identification.
7. Can be automated, or semi-automated using liquid sample handling devices, thereby further reducing “hands-on” time.

#### Solid-Phase Adherence Methods

Two forms of solid-phase adherence assays are available for red cell serologic testing. For direct tests, antibody is fixed to wells of a microplate, and red cells are added (eg, anti-A and anti-B for direct testing of donor/recipient ABO typing). Following centrifugation, red cells expressing the corresponding antigen will efface across the well; red cells lacking the antigen will pellet to the bottom of the well.

For indirect tests, (eg, for detecting unexpected antibodies to red cell antigens) red cell membranes are affixed to microplate wells; test serum or plasma are added and the plates washed to remove unbound globulins. Indicator red cells, which are coated with anti-IgG, are then added and the plates are centrifuged. The indicator red cells efface across the well in a positive test, and pellet to the center of the well in a negative test.<sup>27</sup> An overview of a solid-phase adherence assay for antibody detection is depicted in Fig 5-7.

#### Quality Assurance for Antiglobulin Tests

All negative IATs must be validated using IgG-coated red cells.<sup>14,16(p44)</sup> The agglutination of these red cells confirms that

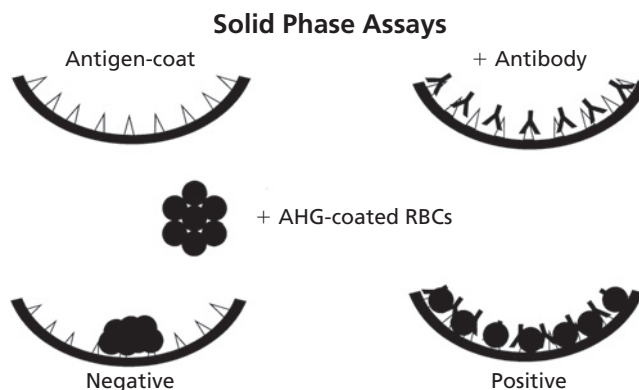


Figure 5-7. Solid-phase adherence assays.

negative tests for unexpected antibodies are not caused by either inadequate washing before the addition of antiglobulin reagent, or inactivation of the antiglobulin reagent by contamination with human serum. This testing is applicable to tube tests; for gel and solid-phase assays, the control system specified by the manufacturer should be used.

#### Automated Pretransfusion Testing

In recent years, several automated systems for pretransfusion testing have received US market approval from the Food and Drug Administration (FDA). They are particularly suited for large hospital-based transfusion services, and include:

1. Automated gel column technology (ProVue, Ortho Clinical Diagnostics, Raritan, NJ).
2. Automated liquid microplate and solid-phase adherence technologies (Galileo, Immucor, Norcross, GA).
3. Microplates containing dried antisera for ABO/Rh typing and a modified solid-phase method for antibody detection (Tango, Biotest Diagnostics, Denville, NJ).

These fully automated systems perform sample and reagent pipetting and subsequent analysis of agglutination reactions. Standard features include positive sample identification, process control through automated documentation of reagent lot numbers and expiration dates, batch and random access operating modes, STAT sample interrupt, and image analysis. The test results can be transmitted via an interface into the laboratory computer system.

The benefits of automation include technologist time savings, positive sample identification, standardized testing (compliance with current good manufacturing practice), increased accuracy, and increased workload capacity. These have to be weighed against the costs for the instrument, reagents, and maintenance, as well as the time required for validation and training.

#### Reagent Red Cells for Antibody Detection

The FDA<sup>28</sup> currently mandates that sets of reagent red cell samples licensed for use in pretransfusion antibody detection tests carry C, c, D, E, e, Fy<sup>a</sup>, Fy<sup>b</sup>, Jk<sup>a</sup>, Jk<sup>b</sup>, K, k, Le<sup>a</sup>, Le<sup>b</sup>, P<sub>1</sub> M, N, S, and s antigens. Such red cells must not be pooled.<sup>16(p43)</sup>

It is impossible to find a single donor with red cells that carry all of the above antigens because adults rarely, if ever, have strong expression of both  $\text{Le}^a$  and  $\text{Le}^b$  on their red cells. Thus, reagent red cells for antibody detection are available commercially as sets of either two or three samples. The Rh phenotypes of red cells used in two-sample sets are  $\text{R}_1\text{R}_1$  ( $\text{D}+\text{C}+\text{c}-\text{E}-\text{e}+$ ) and  $\text{R}_2\text{R}_2$  ( $\text{D}+\text{C}-\text{c}+\text{E}+\text{e}-$ ). In three-sample sets, an  $\text{rr}$  ( $\text{D}-\text{C}-\text{c}+\text{E}-\text{e}+$ ) sample is provided, in addition to  $\text{R}_1\text{R}_1$  and  $\text{R}_2\text{R}_2$  red cells. Use of three red cell samples facilitates the inclusion of red cells from individuals homozygous for particular blood group genes. Such red cells tend to have a stronger expression of an antigen when compared to red cells from individuals heterozygous for the same gene; this phenomenon is known as dosage. It is easier to find double-dose expression of blood group antigens among three reagent red cell samples than two samples; however, use of three samples increases the workload for antibody detection by 50% and rarely affords detection of significant alloantibodies that are not detected with two reagent red cell samples.<sup>29</sup>

### Further Options

Table 5-7 shows changes in pretransfusion testing practices within the United States from 2001 to 2004.<sup>30,31</sup> Of note is the increased use of gel technology for both ABO/Rh typing and antibody detection. Not shown (2004 survey did not include) are practices that are considered redundant, including the following:

1. Use of polyspecific AHG reagents. These contain anticomplement activity in addition to anti-IgG, and facilitate detection of some antibodies that coat red cells with complement components including, notably, anti-Jk<sup>a</sup> and anti-Jk<sup>b</sup>. Failure to detect such antibodies may lead to acute intravascular destruction of antigen-positive red cells.<sup>8</sup> However, use of polyspecific AHG also facilitates detection of IgM complement-binding autoantibodies that are of no clinical significance.<sup>24</sup> Many of these antibodies are autoantibodies directed toward the I antigen, and are found naturally in virtually all normal adult human sera.<sup>8</sup>

2. Room temperature incubation to detect agglutinating alloantibodies, as well as the detection of IgM autoantibodies. Antibodies that do not react at body temperatures rarely, if ever, cause significant destruction of transfused incompatible red cells.<sup>8</sup> While room temperature incubation of tests was widely used through the early 1980s, many laboratories have since abandoned the practice.<sup>30</sup>

3. The use of a microscope to examine serologic tests. Examination for agglutination may be macroscopic, performed using an illuminated concave mirror, or microscopic. The latter is rarely necessary in routine practice; indeed, such critical examination of serologic tests can result in incorrectly recording negative tests as positive.<sup>17,18</sup>

4. Inclusion of an autocontrol (AC) as part of pretransfusion testing. The AC consists of testing the patient's serum against his or her own red cells under the same conditions as those to which screening tests for unexpected antibodies are subjected. This test, or the DAT, is performed to detect globulins bound to

the patient's red cells in vivo. Such in-vivo coating of red cells occurs in patients with autoimmune hemolytic anemia or hemolytic disease of the fetus and newborn, and may also be seen following therapy with certain drugs or transfusion with incompatible blood.<sup>14</sup> Moreover, a positive DAT may be the earliest manifestation of an alloimmune response to a previous, recent transfusion.<sup>32</sup>

Inclusion of the DAT/AC as part of routine pretransfusion testing is no longer advocated; in the absence of detectable serum antibodies, the predictive value of a positive DAT is so low (0.29%) that routine testing is not cost effective.<sup>32</sup> However, the DAT/AC is a good predictive test for immune-mediated hemolysis when performed on samples from patients with clinical manifestations of hemolytic anemia.<sup>33</sup>

### The Principles of Antibody Identification

When unexpected antibodies are present, as indicated by positive screening tests, they must be identified. At a minimum, this involves testing the patient's serum against a panel of fully phenotyped reagent red cell samples as well as the patient's own cells. A typical panel is shown in Table 5-8, which also includes the results of antibody identification tests with a serum containing a mixture of anti-M and anti-K. It should be noted that the tests performed in this example do include a reading for direct agglutination after room temperature incubation and after incubation at 37°C. The results of tests with ficin-treated red cells are also displayed. This example will be used to illustrate a process by which the antibody specificities can be ascertained whenever there are reactive and nonreactive red cell samples. A typical approach follows:

1. The reactions of the AC are examined. Alloantibodies, by definition, should not react with the red cells of the antibody producer. When the AC is reactive, autoantibodies may be present or the AC may react because alloantibodies have formed to recently transfused red cells that are still circulating in the recipient. In other cases, both auto- and alloantibodies may be present. Therapy with certain drugs such as intravenous penicillin or a cephalosporin can also cause the DAT/AC to be positive.<sup>14</sup> The AC is negative in the case shown in Table 5-8, so autoantibodies likely are not present.

2. The graded reaction strengths are examined. If all positive tests are equally reactive, then only a single antibody may be present. If there are no negative tests with reagent red cells but the AC is nonreactive, the presence of antibody to a high-prevalence antigen should be considered. Variability in reaction strength may be an indication of dosage; ie, the antibody reacts stronger with red cells from homozygotes (double-dose) than with red cells from heterozygotes (single-dose). Except for antibodies to D,  $\text{P}_1$ , and  $\text{Xg}^a$  antigens, most blood group antibodies manifest dosage. There is variable expression of  $\text{P}_1$  and  $\text{Xg}^a$  antigen on red cells from different donors, but this is unrelated to their zygosity. Given the varying degrees of reactivity of positive red cell samples in Table 5-8, more than one antibody appears to be present and  $\text{M}+\text{N}-$  red cells react significantly stronger than  $\text{M}+\text{N}+$  red cells.

3. A process of crossing out is undertaken. This process involves evaluating the antigens present on nonreactive red cells. Only

**Table 5-8.** Results of Tests between a Panel of Reagent Red Cells and a Serum Containing Anti-M and Anti-K

| PANEL   | RH                            |   |   |                |   |   |   | MNS |   |   |   | P1             | LE              |                 | KEL |   |                 |                 | JK              |                 | FY              |                 | XG              |  | LISS |    |     | FICIN |     |    |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|---------|-------------------------------|---|---|----------------|---|---|---|-----|---|---|---|----------------|-----------------|-----------------|-----|---|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|--|------|----|-----|-------|-----|----|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|
|         | D                             | C | c | C <sup>w</sup> | E | e | f | M   | N | S | s | P <sub>1</sub> | Le <sup>a</sup> | Le <sup>b</sup> | K   | k | Kp <sup>a</sup> | Js <sup>a</sup> | Jk <sup>a</sup> | Jk <sup>b</sup> | Fy <sup>a</sup> | Fy <sup>b</sup> | Xg <sup>a</sup> |  | RT   | 37 | IAT | 37    | IAT |    |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 1       | r'r                           | 0 | + | +              | 0 | 0 | + | +   | + | 0 |   | 0              | 0               | +               | 0   | + | 0               | 0               | +               | +               | 0               | +               | +               |  | 1    | 3+ | 1+  | 0     | 0   | 0  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 2       | R <sub>1</sub> R <sub>1</sub> | + | + | 0              | 0 | 0 | + | 0   | + | + | + | +              | +               | 0               | 0   | + | 0               | 0               | +               | +               | +               | +               | +               |  | 2    | 0  | 0   | 0     | 0   | 0  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 3       | R <sub>1</sub> R <sub>1</sub> | + | + | 0              | + | 0 | + | 0   | 0 | + |   | +              | 0               | +               | +   | + | 0               | 0               | +               | +               | +               | 0               | +               |  | 3    | 4+ | 3+  | 3+    | 0   | 3+ |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 4       | R <sub>2</sub> R <sub>2</sub> | + | 0 | +              | 0 | + | 0 | 0   | + | + |   | +              | 0               | +               | 0   | + | 0               | 0               | 0               | +               | +               | +               | +               |  | 4    | 0  | 0   | 0     | 0   | 0  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 5       | r'r                           | 0 | 0 | +              | 0 | + | + | +   | + | 0 | + | 0              | +               | 0               | +   | 0 | +               | 0               | 0               | +               | +               | 0               | +               |  | 5    | 4+ | 3+  | 3+    | 0   | 0  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 6       | rrV                           | 0 | 0 | +              | 0 | 0 | + | +   | + | 0 | + | +              | 0               | 0               | 0   | + | 0               | 0               | 0               | +               | 0               | +               | +               |  | 6    | 3+ | 1+  | 0     | 0   | 0  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 7       | rr                            | 0 | 0 | +              | 0 | 0 | + | +   | 0 | + |   | +              | 0               | +               | +   | 0 | 0               | 0               | 0               | +               | +               | 0               | 0               |  | 7    | 0  | 0   | 4+    | 0   | 4+ |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 8       | rr                            | 0 | 0 | +              | 0 | 0 | + | +   | + | + |   | +              | +               | 0               | 0   | + | 0               | 0               | +               | 0               | +               | +               | 0               |  | 8    | 3+ | 1+  | 0     | 0   | 0  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 9       | rr                            | 0 | 0 | +              | 0 | 0 | + | +   | + | 0 |   | +              | 0               | +               | 0   | + | 0               | 0               | +               | 0               | 0               | +               | +               |  | 9    | 4+ | 3+  | 3+    | 0   | 0  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 10      | rr                            | 0 | 0 | +              | 0 | 0 | + | +   | + | + |   | 0              | +               | 0               | +   | + | 0               | 0               | +               | +               | +               | 0               | +               |  | 10   | 3+ | 1+  | 3+    | 0   | 3+ |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 11      | Ror                           | + | 0 | +              | 0 | 0 | + | +   | 0 | 0 |   | +              | 0               | +               | 0   | + | 0               | +               | +               | +               | 0               | 0               | 0               |  | 11   | 3+ | 1+  | 0     | 0   | 0  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| PATIENT |                               |   |   |                |   |   |   |     |   |   |   |                |                 |                 |     |   |                 |                 |                 |                 |                 |                 |                 |  |      |    |     |       |     |    |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
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|         |                               |   |   |                |   |   |   |     |   |   |   |                |                 |                 |     |   |                 |                 |                 |                 |                 |                 |                 |  |      |    |     |       |     |    |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|         |                               |   |   |                |   |   |   |     |   |   |   |                |                 |                 |     |   |                 |                 |                 |                 |                 |                 |                 |  |      |    |     |       |     |    |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|         |                               |   |   |                |   |   |   |     |   |   |   |                |                 |                 |     |   |                 |                 |                 |                 |                 |                 |                 |  |      |    |     |       |     |    |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |

reagent red cell samples 2 and 4 of Table 5-8 are nonreactive. Antibodies to any of the antigens (D, C, c, E, e, N, S, s, P<sub>1</sub>, Le<sup>a</sup>, Le<sup>b</sup>, Jk<sup>a</sup>, Jk<sup>b</sup>, Fy<sup>a</sup>, Fy<sup>b</sup>, and Xg<sup>a</sup>) present on these two cell samples can be eliminated from initial consideration. This leaves only antibodies to M, K, C<sup>w</sup>, f, Kp<sup>a</sup>, and Js<sup>a</sup>. However, C<sup>w</sup>, Kp<sup>a</sup>, and Js<sup>a</sup> are low-prevalence antigens and are not likely to have been present on the reagent red cells used for antibody detection. Similarly, f antigen will not be present on a two-sample screening set of R<sub>1</sub>R<sub>1</sub> and R<sub>2</sub>R<sub>2</sub> cells. Thus, a provisional specificity of anti-M and anti-K can be assigned to this serum.

4. The test phase at which reactivity is observed is evaluated. Antibodies that are usually IgM (see Table 5-2), such as anti-M, -N, -Le<sup>a</sup>, and -P<sub>1</sub>, react as direct agglutinins at room temperature. They do not react solely by an IAT, although there may be carry-over of direct agglutination that can be observed in antiglobulin tests. Similarly, those antibodies that are usually IgG (eg, anti-Rh, -K, -Fy, -Jk, -S) react preferentially by an IAT. However, IgM antibodies of these specificities can be encountered, especially during the early stages of the immune response.

If hemolysis of reagent red cells has been observed, a complement binding antibody such as anti-Le<sup>a</sup> or anti-Jk<sup>a</sup> may be present. This complement-binding activity is often enhanced in tests with ficin-treated red cells, and in the absence of direct lysis of test red cells can best be observed through the use of polyspecific AHG.

With the case under discussion, the anti-M appears to react best at room temperature and the anti-K reacts best by IAT. This is consistent with the anti-M being IgM, although many examples of anti-M do have an IgG component,<sup>9</sup> while the anti-K is most likely IgG (see Table 5-2). The anti-f does not appear to be present because f-positive cell samples 1, 6, 8, and 11 are nonreactive by IAT.

5. The results of tests with enzyme-treated red cells, if performed, are evaluated. Knowledge of the anticipated behavior of certain antibodies with enzyme-treated red cells can be very helpful when identifying antibodies—especially when dealing with

sera containing mixtures of alloantibodies. Antigens of the MNS, FY, and XG systems are cleaved by treatment of red cells with proteolytic enzymes such as papain or ficin. The high-prevalence antigens Ch, En<sup>a</sup>, JMH, In<sup>b</sup>, Rg, Yt<sup>a</sup>, and some Ge antigens are also cleaved by proteolytic enzymes.<sup>9</sup> Negative or weak reactions are observed when antibodies to these antigens are tested with ficin or papain treated red cells. In contrast, treatment of red cells with proteolytic enzymes enhances their reactivity with Rh antibodies, as well as complement-binding antibodies such as anti-Le<sup>a</sup>, -P<sub>1</sub>, and -Jk<sup>a</sup>.

With the case under discussion, the double-dose M-positive red cell samples (3, 5, 9) react in LISS tests at 37°C and by IAT. These cells are nonreactive when pretreated with ficin, which of course is expected for anti-M. In contrast, K antigen is not affected by ficin treatment, so there is no difference in the IAT reactions of ficin and LISS tests with cell samples 3, 7, and 10. Anti-f can now be completely eliminated from consideration because if present, it should have reacted with all the K-negative ficin-treated cells from donors with *r* (*ce*) haplotype.

6. The data are reviewed to ensure that all other antibodies that must be excluded (by virtue of the FDA's requirements for reagent red cells) are not present in the serum. In this case, this was accomplished in Step #3. The fact that antibodies to C<sup>w</sup>, Js<sup>a</sup>, and Kp<sup>a</sup> could be present should not be of concern; they are no more likely to be present in this case than they are in patients with negative screening tests for unexpected antibodies.

Some workers believe that exclusion tests should be performed with double-dose red cells. Their rationale appears to be based on the notion that if a patient has made one alloantibody they are likely to make another—more so than a nonalloimmunized patient is likely to make their first antibody following transfusion. To detect newly forming antibodies, they advocate the use of red cells from apparent homozygotes. However, there are no data to support this notion.

Use of double-dose red cells for pretransfusion antibody detection is not required by either the FDA<sup>28</sup> nor recommended by the AABB<sup>14</sup> (see requirements for reagent red cells). Reagent manufacturers do provide red cells with double-dose expression of Rh antigens, and some workers have established institutional policies regarding the required expression of other antigens on these cells. It would seem appropriate to use the same policies for exclusion purposes whenever practical; ie, ensure absence of anti-Jk<sup>a</sup> with Jk(a+b-) cells if such apparent double-dose expression is required for antibody detection. It would not, however, be practical to exclude anti-E in a patient found to have anti-D using D-negative cells carrying double-dose expression of E, because this would require use of rare r"r" cells.

7. The data are subjected to statistical analysis. Before final conclusions can be made regarding antibody specificity, there must be sufficient negative test results with red cells that lack the corresponding antigen and sufficient positive test results with red cells that carry that antigen. To obtain a confidence level of >95% ( $p = 0.05$ ), there should be at least three nonreactive antigen-negative red cell samples and at least three reactive antigen-positive red cells. This requirement has been met for both the anti-M and the anti-K of Table 5-8.

8. The patient's red cells lack the corresponding antigen. As discussed earlier, this is fundamental to the formation of alloantibodies following transfusion or pregnancy. The red cells from the patient of Table 5-8 should be tested with anti-M and anti-K. They should lack both M and K antigens. There are, however, exceptional cases of alloantibody formation when the corresponding antigen appears to be present on the autologous red cells. Most notably, this is seen with Rh-positive individuals of the partial-D phenotype who make antibody to the portions of D antigen that are absent from their red cells.

The above illustration represents but a basic approach to antibody identification. More complex cases involving autoantibodies, multiple alloantibodies, mixtures of both auto- and alloantibodies and antibodies to high-prevalence antigens will require the resources of an immunohematology reference laboratory. The interested reader is referred elsewhere<sup>14,19</sup> for information regarding the investigation of complex antibody problems.

## Donor-Recipient Testing

### Selection of Blood for Transfusion

#### ABO and Rh

Red Blood Cells (RBCs) and Whole Blood selected for transfusion must be compatible with the serum of the intended recipient. To avoid the hemolytic and often fatal consequences of an ABO-mismatched transfusion, red cells carrying A and/or B antigens should not be transfused to a patient unless the patient's red cells also carry those antigens. Group O individuals should receive group O red cells, but AB individuals can receive red cells of any ABO type. Rh-negative individuals, particularly females of childbearing potential, should receive Rh-negative blood. Rh-positive individuals may receive blood of either Rh type.<sup>14</sup>

### Unexpected Antibodies

When the identified antibodies are known to cause accelerated destruction of transfused incompatible red cells, blood selected for transfusion should be shown to lack the corresponding antigen or antigens.<sup>14,16(p45)</sup> This entails testing donor units with reagent antisera that are available commercially or prepared in-house from previously investigated samples. Examples of potentially significant antibodies include those directed toward antigens of the RH, JK, KEL, and FY systems, and the S and s antigens of the MNS system, as well as most other antibodies active at 37°C and/or by an IAT (see Table 5-2). When antibodies with specificities directed toward M, N, P<sub>1</sub>, and LE antigens are present, particularly when the antibodies react best at or below room temperature, blood selected for transfusion need only be shown to be compatible by IAT following 37°C incubation; demonstrating that compatible units lack the relevant antigen(s) is not required.<sup>34,35</sup> In instances when autoantibodies are present, least incompatible units should be selected for transfusion once it has been established that the autoantibody is not masking a concomitant, clinically significant alloantibody.<sup>33</sup>

### Serologic Crossmatch

Before whole blood or red cells are administered, except in an emergency, a major crossmatch must be performed. This usually entails tests between donor red cells selected for transfusion and the prospective recipient's serum or plasma sample that was used for ABO, Rh, and antibody detection tests. The methods used should be capable of detecting ABO incompatibility and include an IAT. However, in the absence of unexpected antibodies (and absence of records of prior detection of such antibodies) in the intended recipient's serum, only testing to detect ABO incompatibility is required.<sup>16(p46)</sup>

### Antiglobulin Crossmatch

When clinically significant unexpected antibodies are present, or a patient's records indicate that such antibodies have been detected previously, blood selected for transfusion must be tested with the patient's serum or plasma by an IAT. Any of the methods described earlier for antibody detection can be used. An antiglobulin crossmatch can also be performed routinely on patients with nonreactive screening tests for unexpected antibodies. This will detect ABO incompatibility and may detect unexpected antibodies that were missed in pretransfusion screening tests. Unexpected antibodies to low-incidence antigens and antibodies manifesting dosage may be detected in this manner, as may antibodies missed in screening tests because of technical error. However, the predictive value of a positive IAT crossmatch following nonreactive screening tests for unexpected antibodies is sufficiently low that many large hospital transfusion services do not perform an IAT crossmatch except as required when unexpected antibodies are present or there are records of such antibodies.<sup>30,31</sup>

### Detection of ABO Incompatibility

Only a procedure for detecting ABO incompatibility is required when screening tests for unexpected antibodies are negative and there is no record of the patient having had such antibodies in

the past.<sup>16(p46)</sup> Detection of ABO incompatibility can be done serologically or, in certain situations, electronically through the use of computers. An immediate-spin crossmatch between the prospective recipient's serum or plasma and donor red cells suspended in EDTA-saline (to prevent false-negative results from prozone by complement-fixing high-titer anti-A and -B) is an acceptable serologic method for the detection of ABO incompatibility.<sup>36</sup> Alternatively, the ABO groups of both the donor units and a blood sample from the intended recipient can be confirmed immediately before the units are released for transfusion.

### **Electronic Crossmatch**

With the emergence of blood bank information systems, laboratories are beginning to use the integrity of computer software to detect ABO incompatibility between the sample submitted for pretransfusion testing and the donor unit selected for transfusion.<sup>37</sup> This computer or electronic crossmatch (EXM) replaces the immediate-spin test for detecting ABO incompatibility. Currently, an EXM is performed in some 2% of North American facilities,<sup>30,31</sup> and in other countries (Canada, Sweden, Australia, United Kingdom, Hong Kong).<sup>38-42</sup>

An EXM may be used instead of an immediate-spin crossmatch provided that:

1. The computer system has been validated on-site to ensure that only ABO-compatible red-cell-containing components have been selected for transfusion.
2. No clinically significant antibodies are detected in the recipient's serum/plasma and there is no record of previous detection of such antibodies.
3. There are concordant results of at least two determinations of the recipient's ABO type on record, one of which is from a current sample.
4. The system contains the donor unit number, the component name, its ABO/Rh type and the interpretation of confirmatory tests; and recipient information and ABO/Rh.
5. A method exists to verify the correct entry of data before release of blood or components.
6. The computer contains logic to alert the user to discrepancies between donor unit labeling and confirmatory test interpretation, and to ABO incompatibilities between the recipient and the donor unit.
7. The donor unit blood type has been confirmed serologically using red cells from an attached segment.

These requirements are promulgated in *AABB Standards*,<sup>16</sup> which should be consulted for the precise wording. Table 5-9 summarizes the FDA requirements for facilities wishing to implement an electronic crossmatch.<sup>43</sup> Flow diagrams for EXM processes are depicted in Fig 5-8. Information on implementing an EXM is available elsewhere.<sup>44-46</sup>

### **Advantages of the EXM**

The advantages of implementing an EXM are as follows:

1. Technologist time savings. This is the major advantage, and equates to approximately 2.5 minutes per unit crossmatched.<sup>37</sup>

2. Reduced sample volume requirements. A 5- to 7-mL sample is more than adequate, and can be used for EXM for at least 30 days after collection provided other pretransfusion tests are performed soon after collection. The latter greatly facilitates same-day admission programs.

3. Reduced sample handling. Significant reduction in exposure to biohazardous materials results from elimination of the need to prepare donor red cell suspensions from attached segments and the need to use the patient's sample for the immediate-spin crossmatch.

4. No unwanted "false"-positive test results. Cold agglutinins, rouleaux, etc, are a frequent cause of a positive immediate-spin crossmatch following a negative antibody screen. These unwanted positive results are avoided with use of an EXM.

5. Improved accuracy (no unwanted "false"-negative test results), unlike the immediate-spin crossmatch, which is prone to yield false-negative results between some 50% of group B sera and eclectic A<sub>2</sub>B red cells.<sup>47,48</sup> The EXM will detect such incompatibilities provided, of course, the patient and donor ABO types are accurately entered into the computer, and the EXM system has been properly designed, validated, and implemented. Moreover, unwanted negative test results associated with potent, prozoning anti-A and anti-B<sup>36</sup> are avoided.

6. Decreased turnaround time, which can lead to a decrease in the crossmatch-to-transfusion ratio.

### **Disadvantages of the EXM**

The disadvantages of implementing an EXM are few. Barriers to implementation include availability of a laboratory information system with software for blood bank applications, the effort required to develop a system that conforms to AABB and FDA requirements, and the on-site validation process that must be undertaken to obtain FDA approval. Consideration should also be given to the fact one cannot bill for performing EXM, but given current health-care reimbursement practices and policies (managed care, diagnosis-related groups), this may not be a real issue. One can, and should, bill for the second ABO and Rh, if required. A current procedural terminology code of 909090 has been assigned to the EXM. This code can be used to track activity, but does not generate a charge.<sup>49</sup>

### **Prior Records Check**

As part of ongoing quality assurance, and for compliance with the *Standards for Blood Banks and Transfusion Services* of the AABB,<sup>16</sup> the results of current ABO, Rh, antibody detection, and compatibility tests must be checked against records of previous tests, if performed. This must be accomplished before blood is released for transfusion, preferably at the time pretransfusion tests are completed. The specific records that must be checked are those for ABO and Rh typing performed within the previous 12 months, and any difficulties in typing, unexpected

**Table 5-9.** FDA Expectations of Facilities Performing an Electronic Crossmatch<sup>43</sup>**STANDARD OPERATING PROCEDURE (SOP)**

Standard operating procedures shall include:

1. Identification of the source(s) of the application software (name of software vendor or developer, the name and version of the software package).
2. A note to the effect that the computer crossmatch function was designed by a commercial vendor or a modification or extended usage under the control of the blood bank or transfusion service.
3. The location(s) where the system will be used.
4. Institution-specific, detailed instructions that address all facets of the procedure; including all circumstances for which the SOP will be used.

**SOFTWARE REQUIREMENTS**

The software will not permit electronic release of blood products unless there are:

1. Records of two determinations of the recipient's ABO group, one of which is from a current sample.
2. A record of a negative test for clinically significant antibodies from a current specimen and no record of previous detection of such antibodies.

**INFORMATION**

The software contains:

1. Donor unit information:
  - a. Unit number.
  - b. Component name.
  - c. ABO and Rh type.
  - d. The interpretation of the ABO confirmatory test (for units collected, processed and labeled by a separate facility).
2. ABO and Rh type of the recipient.
3. Detection of ABO incompatibility:
  - a. The complete decision table is defined in the software; eg, expected response for each possible combination of recipient/donor ABO combinations.
  - b. Appropriate restrictions based on component type are defined in the software; eg, most facilities will use decision tables to approve/reject release of a number of different red cell products (decision tables must be validated for each product code).
4. Logic to alert the user to:
  - a. Missing required information.
  - b. Donor unit labeling and confirmatory test interruption do not match.
  - c. ABO incompatibilities between the recipient and the donor unit.
  - d. Any other user-defined criteria.

**VALIDATION**

1. The validation design must cover both "automated" software decision-making and decisions made "manually" by users following the SOP.
2. The validation must be performed on site using the same hardware and software that will be employed in routine use.
3. Validation documentation includes:
  - a. Testing strategies.
  - b. Test acceptance and completion criteria.
  - c. Copies of the documentation and testing results.
4. There is a method to review and validate entry of data before it can be used in the decision process. For manual entry this may be double entry of data or an affirmation response after reviewing on-screen display in addition to initial entry.
5. Validation testing must include:
  - a. At least one case of each possible combination of decision variables.
  - b. A reasonable number of cases where one or more required data elements are missing.
  - c. A reasonable number of challenges for the selection of blood and/or components that do not meet specified criteria.
6. If the decision tables govern issues other than ABO (eg, donor/recipient Rh match), they must be adequately tested in the validation protocol.

antibodies, severe adverse reactions to transfusion, and any special transfusion requirements. Any discrepancies between past and present ABO and Rh typing results must be thoroughly investigated; the most likely explanation is that the present sample is not from the same individual whose blood was tested previously. Further, even in the absence of detectable unexpected antibodies in the current sample, a record of such unexpected antibodies in previous samples must be taken into consideration when selecting and crossmatching blood for present and future transfusions.

**Labeling**

Before blood is released for transfusion the container shall have an affixed label or tie tag indicating the recipient's first and last names, unique identification number, the donor unit number, and the interpretation of compatibility tests, if performed.<sup>16(p50)</sup> The unit must be inspected visually before release; if any abnormality in color or appearance is noted, the unit should not be issued. A record should be made of this inspection. The expiration date should also be checked to avoid issuing an out-dated unit.<sup>14</sup>

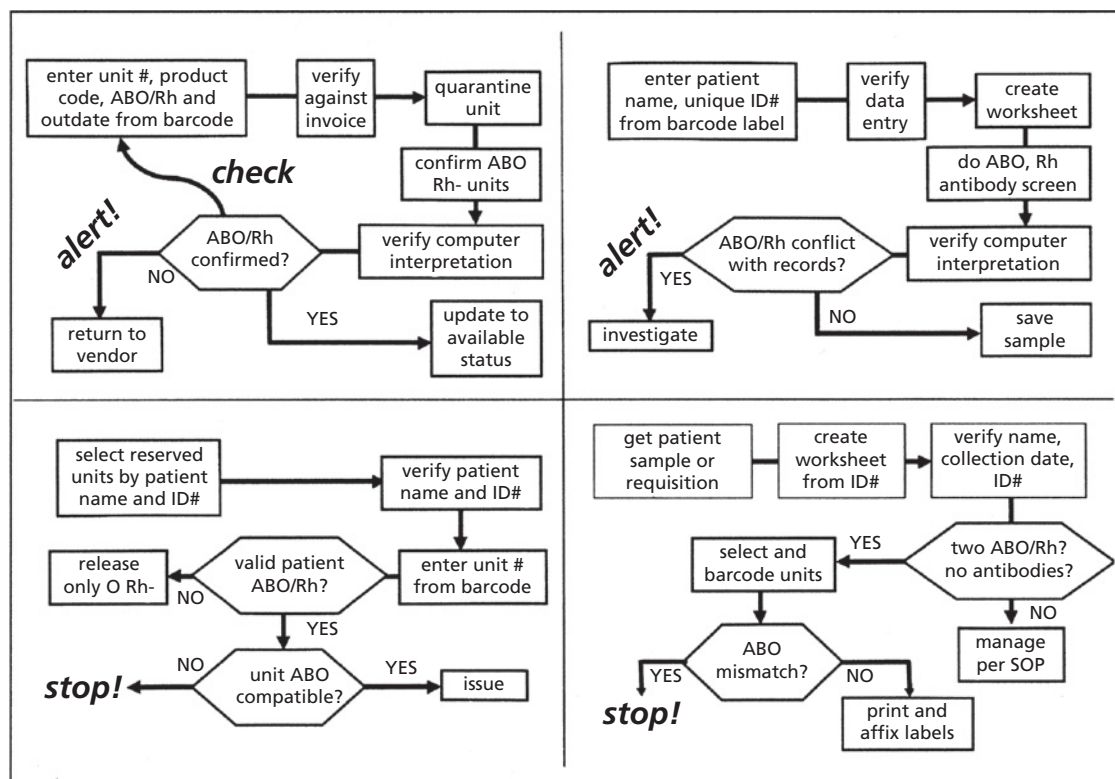


**Figure 5-8.** Flow diagrams for the electronic crossmatch. From the top left: process for donor unit entry and confirmatory ABO/Rh typing; process for initial patient ABO/Rh typing and detecting unexpected antibodies; electronic crossmatch process; dispense (release) process.

### Issue

At the time blood and blood components are issued, there must be a final check of records maintained in the transfusion service.<sup>16(p50-51)</sup> These records shall include:

1. The recipient's name and unique identification number; the ABO group and Rh type of the recipient, if required.
2. The donor unit number, or pool identification number (for platelets and cryoprecipitate); and the ABO group and Rh type of the donor.
3. The interpretation of compatibility tests, if performed.
4. The date and time of issue.

Documentation that this process occurred can be facilitated by use of a log book or the information system, in which the name of the person performing the release check can be recorded.<sup>14</sup>

### Emergency Release

When a patient's ABO group is not known, only group O Red Blood Cells can be issued.<sup>16(p52)</sup> For female patients of childbearing potential, these should also be Rh-negative. If the ABO group and Rh type have been determined on a current sample, type-specific Whole Blood or ABO-compatible RBCs may be issued. The container label or tie tag should indicate that compatibility testing was not completed at the time the unit was released. Testing should be completed expeditiously, and the records should contain a statement, signed by the requesting physician,

indicating the need for transfusion before completion of compatibility testing.

### Bedside Check

Before administering blood, the physician's written order should be reviewed to verify the request for transfusion.<sup>14</sup> The transfusionist who administers the blood is responsible for this and for performing a final errors check. Verification of the following information must occur<sup>16(p53-54)</sup>:

1. *Recipient Identification:* The name and identification number on the patient's wristband must be identical to the name and number on the form attached to the unit.
2. *Unit Identification:* The unit number on the blood container must match the unit number on the transfusion form.
3. *ABO/Rh:* The ABO and Rh type on the donor unit primary label must agree with that recorded on the transfusion form.
4. *Expiration Date:* The expiration date of the unit should be checked and the unit verified as acceptable for transfusion.

### Hemovigilance and Pretransfusion Testing

Blood transfusion subjects a patient to significant risks, ranging in severity from febrile nonhemolytic reactions and red cell alloimmunization to disease transmission and death from bacterial contamination or transfusion of grossly incompatible blood. The latter can prove fatal, and while considerable resources have been spent

in recent decades to reduce infectious disease transmission, only recently has the issue of “errors in transfusion” been addressed. These errors in transfusion care stem from the following causes:

1. Wrong blood in tube, or WBIT, resulting from misidentification of the patient at time of pretransfusion sample collection or mislabeling of the collection tube.
2. Inappropriate transfusion; ie, exposing patients to the hazards of transfusions that they did not need.
3. Mistransfusion; ie, transfusion of the wrong unit or transfusion to the wrong patient.

Adherence to standard operating procedures mandated at an institutional level and use of dedicated phlebotomy service personnel to collect pretransfusion samples can decrease the incidence of WBIT (Friedman BA, personal communication). To eliminate errors caused by WBIT, some facilities require concordant results on two independently collected samples before release of non-group-O red cell products. Yet others have implemented locking devices (eg, Bloodloc, Novatec Medical, Effingham, IL) that require assignment of a three-letter code to any potential transfusion candidate. This code is printed on the patient’s identification bracelet and transcribed on the sample tube and requisition at time of sample collection. After completion of pretransfusion testing, units of blood are placed into plastic bags, each of which is closed with a combination lock set to the patient’s assigned three-letter code. At the bedside, the transfusion nurse can open the lock only by using the patient’s code. If there is any discrepancy, the blood is returned to the blood bank.

More recently, transfusion services have begun to apply new technologies widely available in commerce to prevent these errors in transfusion.<sup>50</sup> Such technologies include:

1. Barcodes and radio-frequency identification (RFID) technologies, both of which serve to improve the accuracy of sample labeling and the bedside checking process. RFIDs are also capable of constant monitoring of blood product location and storage temperature, thereby improving compliance with good manufacturing practice regulations.
2. Computerized order entry and support systems to improve the decision-making process, including use of nanotechnology sensors that act as barriers to inappropriate transfusions.

Technologies now being implemented in Europe place the entire transfusion process under computerized control.<sup>51</sup> Features of such a process include:

1. Direct order entry, requiring documentation of the reason to transfuse and notification of the ordering physician if the request does not meet established guidelines.
2. Barcode scanning of both phlebotomist and patient identification bracelets at time of sample collection, using a personal digital assistant (PDA) device to produce accurate sample labels.
3. Following processing and compatibility testing, scanning and placement of blood into a computer controlled blood issue refrigerator.
4. Tracking of all movements in and out of the refrigerator. Access to patient-assigned products requires entry of operator and patient identification information.

Following FDA approval and successful validation, it is anticipated that such systems will gain acceptance within the United States.

## Conclusion

Compatibility testing constitutes a quality assurance program designed to detect serologic incompatibility between donor unit and the intended recipient and to prevent both clerical and technical errors that may have serious if not fatal consequences. Assurance of quality requires proper performance of each individual task. There can be no substitute for proper patient identification, proper sample labeling, and proper performance of serologic tests.

## Disclaimer

The author has disclosed no conflicts of interest.

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# 6

## Carbohydrate Blood Groups

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Many important human blood group antigens are glycoconjugates. These antigens are grouped together in this chapter because there are similarities in their biosynthesis, in the human immune response to these antigens, and in the outcome in vivo after transfusion of incompatible blood.

The antigens in the ABH, Lewis, P, and I blood group systems are synthesized by interrelated pathways. These oligosaccharide antigens may exist free in solution. In addition, they may be covalently attached to lipids (ie, ceramides) to form glycosphingolipids, or to polypeptides to form mucins, integral membrane glycoproteins, or soluble glycoproteins. Specific glycosyltransferase enzymes catalyze formation of the relevant glycosidic linkages (ie, the bonds between monosaccharides). Some glycosyltransferases, found in all individuals, form framework structures. The genes encoding other glycosyltransferases are allelically inherited and the resulting enzymes specify the synthesis of variable structures. Because of their variable inheritance and expression, the latter may form immunologically recognized blood group antigens. The absence of particular blood group antigens in certain individuals may result in specific antibody production after stimulation by the foreign antigen. As described below, antigens in the ABH, Lewis, P, and I blood group systems are synthesized on common precursor framework molecules. Competition between genetically inherited blood-group-specific glycosyltransferases results in a rich mixture of antigenic molecules. In addition, a single oligosaccharide may encode several different blood-group antigens.

The immune response to carbohydrate antigens, particularly when presented as repetitive epitopes, is typically thymus independent (for review, see Mond et al<sup>1</sup>). That is, the repetitive, multivalent antigens can directly stimulate B cells to synthesize antibodies without the aid of helper T cells. Thymus-independent immune responses classically produce IgM antibodies, and most antibodies to carbohydrate blood group antigens are of the IgM

class. Individuals lacking a particular carbohydrate blood group antigen on their red cells often have “naturally occurring” IgM antibodies to that antigen in their sera, even if they were not previously exposed to human blood components. Most evidence suggests that these antibodies did not arise spontaneously without prior antigenic stimulation, but rather that cross-reacting antigens in the environment, such as on gut bacteria, stimulate specific IgM production.<sup>2</sup> For example, a particular serotype of *Escherichia coli* synthesizes the blood group B antigen as part of its lipopolysaccharide.<sup>3</sup> In contrast, high-titered IgG antibodies to carbohydrate antigens are found in certain individuals. These IgG antibodies may be induced by a thymus-dependent form of these oligosaccharides, perhaps as individual epitopes on glycoproteins, in which T-cell help leads to an isotype switch from IgM to IgG.

Because antibodies to carbohydrate blood group antigens are predominantly IgM, these decavalent molecules directly agglutinate antigen-positive human red cells without the aid of an antiglobulin reagent. Agglutination by these antibodies in vitro typically improves at temperatures <37°C. Because most IgM molecules directly fix complement, these antibodies can cause immediate intravascular hemolysis after transfusion of incompatible, antigen-positive red cells. In unusual cases, carbohydrate-specific IgG antibodies coat red cells in vivo, leading to extravascular hemolysis after incompatible transfusion. In addition, the latter may cross the placenta, resulting in hemolytic disease of the fetus and newborn.<sup>4,5</sup>

### ABH, Secretor, and Lewis Systems

#### ABH Antigens: Introduction

The ABO blood group system is the most important one with respect to blood transfusion, hematopoietic stem cell transplantation, and solid organ transplantation. Karl Landsteiner was the first to discover human alloantigens by using a conceptually simple experiment (Table 6-1). The red cells of each individual were found either to lack or to have one or both of two antigens, A and B. In addition, the serum of each subject contained “naturally

Table 6-1. ABO Blood Group Antigens and Antibodies

| Blood Group | Red Cell Antigens |   | Serum Antibodies |        |
|-------------|-------------------|---|------------------|--------|
|             | A                 | B | Anti-A           | Anti-B |
| A           | +                 | − | −                | +      |
| B           | −                 | + | +                | −      |
| AB          | +                 | + | −                | −      |
| O           | −                 | − | +                | +      |

occurring” directly agglutinating antibodies that recognized the antigens absent from their own red cells. The modern explanation of this experiment is that cross-reacting carbohydrate structures on environmental agents stimulate thymus-independent production of IgM anti-A and/or anti-B in individuals not tolerant of these antigens. The IgM antibodies then directly agglutinate the corresponding antigen-positive red cells.

Although the ABH antigens (the H antigen is the relevant carbohydrate structure present on group O red cells) are typically described as “blood group antigens” because of their presence on red cells, they are also found on other tissues, and are more appropriately termed “histo-blood group antigens.”<sup>6</sup> In blood they are both membrane bound (eg, on platelets<sup>7</sup>) and soluble (eg, as blood-group-active glycosphingolipids inserted in plasma lipoprotein particles). They also are membrane antigens on such diverse cells as vascular endothelial cells and intestinal, cervical, urothelial, and mammary epithelial cells. Soluble forms are found in various secretions and excretions, such as saliva, milk, urine, meconium, and feces. Their expression in some tissues is developmentally regulated.<sup>8</sup> Despite their wide distribution, genetic inheritance, developmental regulation, and importance in transfusion and transplantation, their normal physiologic function, if any, remains a mystery.

ABH Antigens: Biochemistry

To appreciate the structure and antigenicity of ABH antigens, and their relationship to other blood group systems, it is necessary to understand the underlying biochemistry. Early studies indicated that anti-A, anti-B, and anti-H specifically recognize epitopes composed of the terminal trisaccharides or disaccharides illustrated in Figure 6-1. From these results it was possible to conclude that the A, B, and H antigens are not directly encoded by the corresponding genes; rather, the genes encode glycosyltransferases, commonly called the A, B, and H transferases, or equivalently, the A, B, and H enzymes. The H enzyme is a fucosyltransferase that specifically adds fucose in an α1-2 linkage to a terminal galactose. The A and B enzymes then add N-acetylgalactosamine or galactose, respectively, in an α1-3 linkage to the same terminal galactose. In addition, the substrate for the A and B enzymes is the H antigen sequence; these enzymes do not transfer the relevant sugar to galactose in the absence of (α1-2)-linked fucose. Similarly, the H enzyme does not function if this galactose is

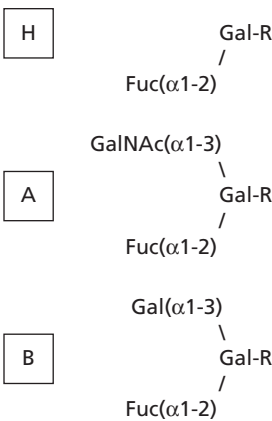
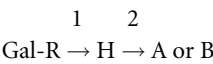


Figure 6-1. Biochemical structures of the immunodominant oligosaccharides of the A, B, and H antigens. Gal = D-galactose; GalNAc = D-N-acetylgalactosamine; Fuc = L-fucose.

already substituted with a different sugar. Thus, the biosynthetic pathway is as follows:



where reaction 1 is catalyzed by the H enzyme and reaction 2 by the A or B enzyme.

The finding that the A and B genes encode glycosyltransferases explains some classical results from the analysis of family pedigrees. In particular, the A and B genes are inherited in a strict mendelian fashion and are dominant compared to O, but A and B are codominant with each other. That is, an individual with the genotype AO (or BO) is phenotypically A (or B), an individual of genotype AB is phenotypically AB, and an individual of genotype OO is phenotypically O. Because the A and B enzymes both use the H antigen as substrate, even the presence of only approximately 50% of these enzyme proteins in an AO (or BO) heterozygote is sufficient to convert the red cells to the corresponding A (or B) phenotype. Similarly, if both the A and B enzymes are present, they each convert approximately 50% of the available H antigen substrate, yielding red cells expressing both the A and B antigens.

Most of the molecular biology of the ABH system has been elucidated over the last 15 years. The allelic A and B genes are located on chromosome 9<sup>9,10</sup> (see Table 6-2). They encode membrane-bound glycosyltransferases containing 354 amino acids (Fig 6-2) and are members of the Glycosyltransferase 6 family cataloged in the CAZY database (<http://www.cazy.org>). Homologues of the A and B genes can be identified throughout evolution, beginning with amphibians.<sup>21</sup> In addition, the A and B enzymes exhibit a great degree of homology and differ at only four amino acid residues, at positions 176, 235, 266, and 268.<sup>15,22</sup> The two adjacent residues near the C-terminus of the protein are critically important in determining their substrate specificity: amino acids 266

and 268<sup>23,24</sup> (Fig 6-2, Table 6-3). A valuable Web site, maintained by the National Center for Biotechnology Information, contains a current catalog of the sequences of the known human blood group antigens, including the glycosyltransferases encoding the ABH system (Blood Group Antigen Gene Mutation Database; <http://www.ncbi.nlm.nih.gov/projects/mhc/xslcgi.fcgi?cmd=bgmute/home>; Blumenfeld and Patraik<sup>25</sup>). The current chapter uses the nomenclature defined in that database.

The explosion of readily available sequence data led to the identification of many variant alleles in the ABH system. For example, to date, more than 100 alleles of the *A* and *B* genes have been identified. Several illustrative variants are described below

**Table 6-2.** Chromosomal Assignment of Cloned Genes in the ABH, Se, Le, I, and P Blood Group Systems

| Gene                      | Gene Product                                                          | Location      | Ref.  |
|---------------------------|-----------------------------------------------------------------------|---------------|-------|
| <i>H</i> ( <i>FUT1</i> )  | ( $\alpha$ 1-2) fucosyltransferase (Fuc-TI)                           | 19q13.3       | 11    |
| <i>Se</i> ( <i>FUT2</i> ) | ( $\alpha$ 1-2) fucosyltransferase (Fuc-TII)                          | 19q13.3       | 11    |
| <i>Sec1</i>               | Pseudogene (nonfunctional; homologue of <i>FUT1</i> and <i>FUT2</i> ) | 19q13.3       | 12,13 |
| <i>Le</i> ( <i>FUT3</i> ) | ( $\alpha$ 1-3/4) fucosyltransferase (Fuc-TIII)                       | 19p13.3       | 11,14 |
| <i>A</i>                  | ( $\alpha$ 1-3) N-acetylgalactosaminyl transferase                    | 9q34.1-q34.2  | 9, 15 |
| <i>B</i>                  | ( $\alpha$ 1-3) galactosyltransferase                                 | 9q34.1-q34.2  | 9, 15 |
| <i>I</i>                  | ( $\beta$ 1-6) N-acetylglucosaminyl transferase                       | 9q21          | 16    |
| <i>P<sup>k</sup></i>      | ( $\alpha$ 1-4) galactosyltransferase                                 | 22q13.2       | 17    |
| <i>P<sup>I</sup></i>      | ( $\alpha$ 1-4) galactosyltransferase                                 | 22q12.3-q13.1 | 18,19 |
| <i>P</i>                  | ( $\beta$ 1-3) N-acetylgalactosaminyl transferase                     | 3q25          | 20    |

The *A* and *B* genes are allelic. The *H*, *Se*, and *Sec1* genes are encoded at distinct, but closely linked, loci and are not alleles of each other. Although the *H*, *Se*, *Sec1*, *Le*, *A*, *B*, *P*, *P<sup>k</sup>*, and *I* cDNA sequences and some genomic sequences have been determined, neither the *P<sup>I</sup>* gene nor its cDNA have yet been cloned.

and in Tables 6-3 through 6-5. For example, in addition to identifying the original (ie, wild type) *A* and *B* genes (herein denoted *A101* and *B101*; see Table 6-3), molecular methods also identified several fully functional variants of each (ie, *A102*, *A103*, *A104*, *B102*, and *B103*) (Tables 6-4 and 6-5). These variants contain silent nucleotide polymorphisms that do not affect the function of these enzymes.<sup>15,26-30</sup>

A common allele corresponding to blood group O, *O01*, is highly homologous to the *A* and *B* alleles, but contains a single nucleotide deletion near the N-terminus of the protein. This leads to a shift in reading frame and a prematurely terminated translation product that lacks enzymatic activity (Table 6-3).<sup>15</sup> More than 50 other *O* alleles have been identified that do not encode functional glycosyltransferases,<sup>27,28,31-38</sup> including some with missense mutations that abolish enzymatic activity.<sup>31,32,36</sup>

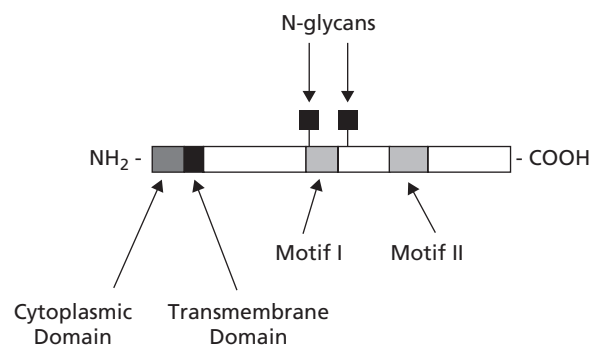


**Figure 6-2.** Structure of a generic blood group-active glycosyltransferase. The enzymes involved in the synthesis of blood-group-active carbohydrate antigens all share a similar overall structure. That is, they are Type II membrane glycoproteins that contain a single N-terminal cytoplasmic domain, a single transmembrane domain, a single large catalytic domain, a variable number of N-glycans, and a variable number of structurally important motifs. In particular, they all share a "DXD" motif involved in binding to a catalytically critical divalent cation (eg, a  $Mn^{+2}$  atom).

**Table 6-3.** Nucleotide and Amino Acid Sequences of the cDNA Derived from the *A*, *B*, and *O* Genes

|                 | Nucleotide Residues |        |       |            |        |        |        |        |        |        |        |        |        |        |        |
|-----------------|---------------------|--------|-------|------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|                 | 188                 | 189    | 220   | 261        | 297    | 526    | 646    | 657    | 681    | 703    | 771    | 796    | 803    | 829    | 930    |
| <b>Alleles</b>  |                     |        |       |            |        |        |        |        |        |        |        |        |        |        |        |
| <i>A101</i>     | G                   | c      | C     | G          | a      | C      | T      | c      | g      | G      | c      | C      | G      | G      | g      |
| <i>B101</i>     |                     |        |       |            | g      | G      |        | t      |        | A      |        | A      | C      |        | a      |
| <i>O01</i>      |                     |        |       | $\Delta$ G |        |        |        |        |        |        |        |        |        |        |        |
| <i>O02</i>      | A                   | t      | T     |            | g      |        | A      |        | a      |        | t      |        |        | A      |        |
| <b>Mutation</b> | 63R→F               | silent | 74P→S | frameshift | silent | 176R→G | 216F→I | silent | silent | 235G→S | silent | 266L→M | 268G→A | 277V→M | silent |

The *A<sub>1</sub>* and *B* transferases are 354 amino acid, Type II, membrane glycoproteins. All sequences in this figure are compared with the *A<sub>1</sub>* sequence encoded by the *A101* allele; blank boxes represent nucleotides identical to the *A101* allele. An uppercase nucleotide indicates that there is an amino acid change induced by the mutation, whereas a lowercase letter indicates that the nucleotide polymorphism is silent at the amino acid level. The frameshift mutation induced by the deletion at nucleotide 261 in *O01* allows for the translation of a significant length of amino acid sequence that differs from the *A101* sequence before an early termination signal at a new stop codon.

**Table 6-4.** Weak Subgroups of A: cDNA Sequences

|                 | Nucleotide Residues |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |            |
|-----------------|---------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|------------|
|                 | 297                 | 467    | 526    | 564    | 646    | 657    | 681    | 703    | 771    | 796    | 803    | 829    | 871    | 930    | 1009   | 1054   | 1059       |
| <b>Alleles</b>  |                     |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |            |
| <i>A101</i>     | a                   | C      | C      | c      | T      | c      | g      | G      | c      | C      | G      | G      | G      | g      | A      | C      | C          |
| <i>A102</i>     |                     | T      |        |        |        |        |        |        |        |        |        |        |        |        |        |        |            |
| <i>A103</i>     |                     | T      |        | t      |        |        |        |        |        |        |        |        |        |        |        |        |            |
| <i>A104</i>     | g                   |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |            |
| <i>A201</i>     |                     | T      |        |        |        |        |        |        |        |        |        |        |        |        |        |        | ΔC         |
| <i>A202</i>     |                     |        |        |        |        |        |        |        |        |        |        |        |        |        |        | T      |            |
| <i>A204</i>     | g                   |        | G      |        |        | t      |        | A      | t      |        |        | A      |        |        |        |        |            |
| <i>A205</i>     |                     | T      |        |        |        |        |        |        |        |        |        |        |        |        | G      |        |            |
| <i>A206</i>     |                     |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        | ΔC         |
| <i>A301</i>     |                     |        |        |        |        |        |        |        |        |        |        |        | A      |        |        |        |            |
| <i>A302</i>     |                     |        |        |        |        |        |        |        |        |        |        | A      |        |        |        |        | ΔC         |
| <i>AX01</i>     |                     |        |        |        | A      |        |        |        |        |        |        |        |        |        |        |        |            |
| <i>AX02</i>     | g                   |        |        |        | A      |        | a      |        | t      |        |        | A      |        |        |        |        |            |
| <i>AX03</i>     |                     |        |        |        | A      |        | a      |        | t      |        |        | A      |        |        |        |        |            |
| <i>Ael01</i>    |                     |        |        |        |        |        |        |        |        |        | ins G  |        |        |        |        |        |            |
| <i>Ael02</i>    |                     | T      |        |        | A      |        | a      |        |        |        |        |        |        |        |        |        |            |
| <i>B101</i>     | g                   |        | G      |        |        | t      |        | A      |        | A      | C      |        |        | a      |        |        |            |
| <b>Mutation</b> | silent              | 156P→L | 176R→G | silent | 216F→I | silent | silent | 235G→S | silent | 266L→M | 268G→A | 277V→M | 291D→N | silent | 337R→G | 352R→W | frameshift |

All sequences in this table are compared with the *A101* allele. The *B101* allele is included for comparison. The frameshift mutation induced by the deletion at nucleotide 1059 in alleles *A201*, *A206*, and *A302* allows for the translation of several additional amino acids beyond the original stop codon, until the translation process encounters a new stop codon. The insertion at nucleotide 803 in the *Ael01* allele induces a frameshift that allows for the translation of a significant length of amino acid sequence that differs from the *A101* sequence before a termination signal at a new stop codon.

**Table 6-5.** Weak Subgroups of B and the B(A) and *cis*-AB Phenotypes: cDNA Sequences

|                 | Nucleotide Residues |        |        |        |        |        |        |        |        |        |        |        |        |
|-----------------|---------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|                 | 297                 | 467    | 526    | 641    | 657    | 669    | 700    | 703    | 796    | 803    | 871    | 930    | 1054   |
| <b>Alleles</b>  |                     |        |        |        |        |        |        |        |        |        |        |        |        |
| <i>A101</i>     | a                   | C      | C      | T      | c      | G      | C      | G      | C      | G      | G      | g      | C      |
| <i>B101</i>     | g                   |        | G      |        | t      |        |        | A      | A      | C      |        | a      |        |
| <i>B102</i>     | g                   |        | G      |        | t      |        |        | A      | A      | C      |        |        |        |
| <i>B103</i>     | g                   |        | G      |        |        |        |        | A      | A      | C      |        | a      |        |
| <i>B301</i>     | g                   |        | G      |        | t      |        |        | A      | A      | C      |        | a      | T      |
| <i>Bx01</i>     | g                   |        | G      |        | t      |        |        | A      | A      | C      | A      | a      |        |
| <i>Bel01</i>    |                     |        | G      | G      | t      |        |        | A      | A      | C      |        | a      |        |
| <i>Bel02</i>    |                     |        | G      |        | t      | T      |        | A      | A      | C      |        | a      |        |
| <i>ABO1cs</i>   |                     | T      |        |        |        |        |        |        |        | C      |        |        |        |
| <i>B(A)01</i>   | g                   |        | G      |        |        |        |        |        | A      | C      |        | a      |        |
| <i>B(A)02</i>   | g                   |        | G      |        | t      |        | G      | A      | A      | C      |        | a      |        |
| <b>Mutation</b> | silent              | 156P→L | 176R→G | 214M→R | silent | 223E→D | 234P→A | 235G→S | 266L→M | 268G→A | 291D→N | silent | 352R→W |

All sequences in this table are compared with the *A101* allele. The *B101* allele is included for comparison. The critical amino acids that distinguish the A and B enzymes are at positions 176, 235, 266, and 268.

These findings using molecular biology not only confirm and extend the results obtained from carbohydrate biochemistry and enzymology, but they also suggest new ways of blood typing.<sup>38-41</sup>

### Antigenic Variants in the ABH System

Some relatively common variations in the ABH system relate to the “strength” of the A antigen on group A red cells. Multiple subgroups of A that have weak expression of the A antigen are known. The red cells of most group A individuals (eg, 80% of Whites) type as  $A_1$  due to their inheritance of one of the  $A^1$  alleles. Most of the remaining group A individuals have weaker expression of this antigen and are denoted  $A_2$ . Other less common subgroups of A (eg,  $A_3$ ,  $A_x$ ,  $A_m$ ,  $A_{el}$ ) have progressively weaker A antigen expression. Many individuals with weak A expression produce an antibody, anti- $A_1$ , which does not agglutinate their own red cells but does agglutinate  $A_1$  red cells. This phenomenon is explained by quantitative and qualitative differences in antigen expression. For example, the number of A antigen sites per red cell varies from approximately 800,000 sites for  $A_1$  cells to 250,000 sites for  $A_2$  cells to 700 sites for  $A_m$  cells.<sup>42</sup> The finding that  $A_2$  individuals can produce  $A_1$ -specific antibodies also implies the existence of qualitative differences. Indeed, biochemical investigations demonstrate that A antigens on  $A_1$  red cells differ from those on cells of the various subgroups of A.<sup>43,44</sup> Many different classes of ABH-active glycosphingolipids have been isolated from human red cells (for example, see Clausen et al<sup>43</sup>) and novel structures continue to be discovered. Examples of relevant structures, provided in Fig 6-3, illustrate that complex oligosaccharides (ie, on Type 3 chains) can contain the A epitope not only as a terminal trisaccharide, but also as an “internal” structure. Thus, one hypothesis is that individuals whose red cells type as  $A_1$  can express all of these classes of structures, whereas those whose red cells type as  $A_2$  lack the

structures built on Type 3 and Type 4 chains; this implies that  $A_1$  antibodies specifically recognize these structures.

The presence of Type 3 chains on  $A_1$  human red cells has another practical implication related to the goal of producing “universal donor” red cells by enzymatically removing the immunodominant GalNAc residues from A-active oligosaccharides.<sup>45</sup> Thus, although many  $\alpha$ -N-acetylgalactosaminidases can remove terminal GalNAc residues, all  $\alpha$ -N-acetylgalactosaminidases identified to date are unable to remove the internal GalNAc residues found on Type 3 chains.<sup>46</sup> Therefore, although it is now possible to perform the enzymatic  $A_2 \rightarrow O$  conversion, the goal of performing the  $A_1 \rightarrow O$  conversion to prepare large numbers of universal donor units currently remains elusive.<sup>46</sup>

More recently, the molecular biological approach has identified mutations in the cDNA and genomic DNA sequences of the A alleles of individuals with red cells expressing weak A activity (Table 6-4).<sup>28,29,47-53</sup> For example, the A transferase in  $A_2$  individuals encoded by the *A201* allele has a 156 P→L missense mutation and a frame shift mutation near the 3'-end of the coding sequence.<sup>49</sup> In addition, some  $A_3$  individuals have an allele, *A301*, containing a 291 D→N mutation in the coding sequence.<sup>48</sup> These types of findings are summarized in Table 6-4. In some cases, transfection studies prove that these mutations result in weakened or variant enzyme activity of the encoded glycosyltransferase (for example, see Yamamoto et al<sup>49</sup>). When the coding sequence is evaluated alone, there is not always a one-to-one correspondence between genotype and phenotype. For example, even within a given family, the red cells of individuals inheriting the *A206* allele can have either the  $A_2$  or  $A_3$  phenotype.<sup>53</sup> In another family, the red cells of individuals inheriting the *A201* allele typed as either  $A_2$  or  $A_x$ .<sup>50</sup> A complete understanding of these results is not yet available, but it may be important to investigate the regulation of expression of

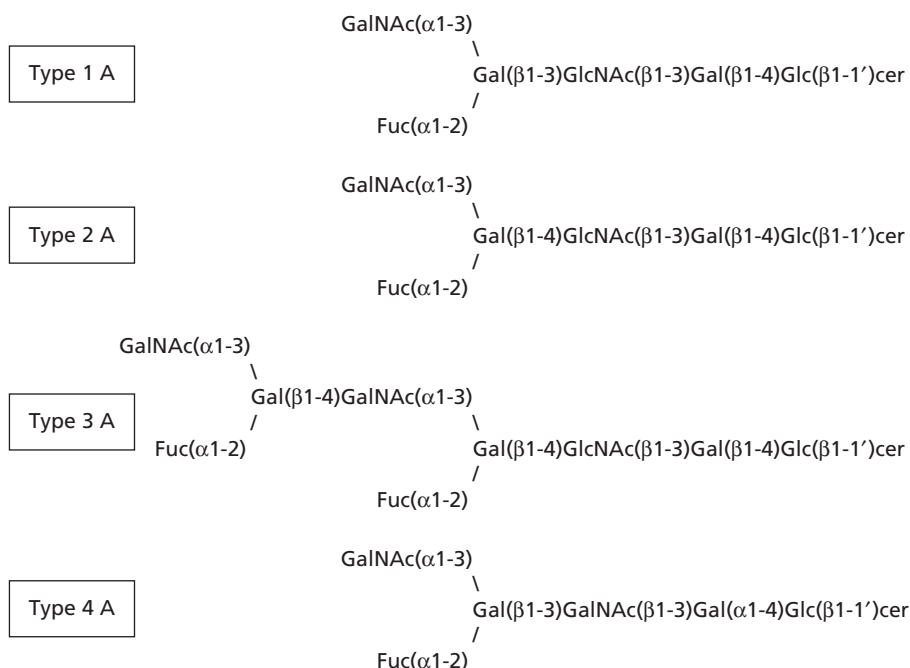


**Figure 6-3.** Biochemical structures of the A antigen-active terminal trisaccharide carried on various types of backbone structures (ie, Types 1-4) attached to a glycosphingolipid. Glc = D-glucose; GlcNAc = D-N-acetylglucosamine; cer = ceramide; Gal = D-galactose; GalNAc = D-N-acetylglactosamine; Fuc = fucose.

these genes in greater detail, particularly by focusing on the promoter and enhancer regulatory regions.<sup>54-57</sup> Finally, the identification of individuals with weak subgroups of A allowed the first successful attempts to transplant “incompatible” A<sub>2</sub> renal allografts into group O recipients.<sup>58</sup>

By analogy with the subgroups of A, “weak” subgroups of B also exist (eg, B<sub>3</sub>, B<sub>x</sub>, B<sub>el</sub>). For example, the gene encoding the B301 allele responsible for the B<sub>3</sub> subgroup has a 352 R → W mutation in the coding sequence that is not found in the wild type B101 allele (Table 6-5).<sup>48</sup>

The B(A) and *cis* AB phenotypes are interesting and unusual variations in the ABH system.<sup>59</sup> In each case, one chromosome in the affected individual apparently encodes for a gene (or genes) that leads to the synthesis of both the A and B antigens. In the B(A) phenotype, the red cells predominantly have B antigens with a small amount of A antigens.<sup>47</sup> In the *cis* AB phenotype, the red cells have equivalent amounts of A and B antigens.<sup>60</sup> Molecular studies demonstrated that the coding sequence of one B(A) transferase, B(A)01, is virtually identical to the wild type B101 allele, except that it lacks the 235 G → S mutation.<sup>47</sup> Thus, at three of the relevant amino acid positions the B(A)01 allele is identical to B101 and at one position it is identical to A101; this allows the resulting glycosyltransferase to create small amounts of A antigen in addition to large amounts of B antigen.<sup>47</sup> In contrast, the sequence of the B(A)02 allele is identical to B101 except for the addition of a 234 P → A mutation (Table 6-5); this mutation is immediately adjacent to one of the four key amino acid residues that confer A or B transferase activity,<sup>61</sup> thereby presumably modifying their function. In one case of *cis* AB, the AB01cs allele has mutations resulting in two amino acid substitutions (Table 6-5).<sup>62</sup> The 156 P → L mutation is identical to that found

in the weak A allele, A201; the 268 G → A mutation is a change that is important for conferring B transferase activity to the resulting enzyme.<sup>23,24</sup> Thus, these changes result in an enzyme that functions as an AB transferase chimera. No evidence for an alternative model, in which crossing-over results in one chromosome that contains *both* an intact A gene and a separate, intact B gene, has been found.

A major recent advance in our understanding of the structure and function of the wild type and variant A and B transferases resulted from the determination of the x-ray crystal structure of the blood group B transferase.<sup>63</sup> This initial structure provided great insight to the mechanism of binding of the nucleotide sugar donor (ie, UDP-Gal) and the oligosaccharide acceptor substrate (ie, the blood group H-active disaccharide). In addition, the availability of this structure allowed for models to be constructed of the A transferase.<sup>64</sup> More recently, mechanistic insight has been gained regarding why the 214 M → R mutation encoded by the *Bel01* gene (Table 6-5) leads to markedly weakened B transferase activity.<sup>65</sup> In particular, the amino acid at position 214 is immediately adjacent to the “DXD” motif at D211-V212-D213 that is critically involved in coordinating the Mn<sup>2+</sup> atom required for enzyme activity. Therefore, this single mutation prevents binding of Mn<sup>2+</sup> and, thereby, markedly diminishes transferase function, an instructive and remarkably beautiful result. Similar types of studies will no doubt yield increasingly important and useful information.<sup>66</sup>

### Secretion of ABH Antigens

The ABH antigens are found not only on red cells, but also in secretions, particularly saliva and plasma. The ability to secrete ABH antigens is genetically inherited: approximately 80% of Whites are

**Table 6-6.** Biochemical Structures of ABH Antigens

| Blood Group | Secretions (Type 1 Chain)                                                                                                                                                       | Red Cells (Type 2 Chain)                                                                                                                                                        |
|-------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| H           | $\begin{array}{c} \text{Gal}(\beta 1-3)\text{GlcNAc-R} \\ \diagup \\ \text{Fuc}(\alpha 1-2) \end{array}$                                                                        | $\begin{array}{c} \text{Gal}(\beta 1-4)\text{GlcNAc-R} \\ \diagup \\ \text{Fuc}(\alpha 1-2) \end{array}$                                                                        |
| A           | $\begin{array}{c} \text{GalNAc}(\alpha 1-3) \\ \diagdown \\ \begin{array}{c} \text{Gal}(\beta 1-3)\text{GlcNAc-R} \\ \diagup \\ \text{Fuc}(\alpha 1-2) \end{array} \end{array}$ | $\begin{array}{c} \text{GalNAc}(\alpha 1-3) \\ \diagdown \\ \begin{array}{c} \text{Gal}(\beta 1-4)\text{GlcNAc-R} \\ \diagup \\ \text{Fuc}(\alpha 1-2) \end{array} \end{array}$ |
| B           | $\begin{array}{c} \text{Gal}(\alpha 1-3) \\ \diagdown \\ \begin{array}{c} \text{Gal}(\beta 1-3)\text{GlcNAc-R} \\ \diagup \\ \text{Fuc}(\alpha 1-2) \end{array} \end{array}$    | $\begin{array}{c} \text{Gal}(\alpha 1-3) \\ \diagdown \\ \begin{array}{c} \text{Gal}(\beta 1-4)\text{GlcNAc-R} \\ \diagup \\ \text{Fuc}(\alpha 1-2) \end{array} \end{array}$    |

secretors and 20% are non-secretors. This trait is inherited as a single locus gene in simple mendelian fashion. The secretor gene (*Se*) is dominant; non-secretor (*se*) is recessive. The terminal carbohydrate sequences of the ABH antigens in saliva and plasma are identical to those on red cells. However, the backbone or framework carbohydrate structures are different. ABH antigens on glycosphingolipids and glycoproteins synthesized by red cell precursors are primarily coupled to framework Type 2 chains (ie, Gal(β1-4)GlcNAc-R); the same antigens on plasma glycosphingolipids and salivary mucins are coupled to framework Type 1 chains (ie, Gal(β1-3)GlcNAc-R) (Table 6-6). Because ABH blood-group-active glycosphingolipids on plasma lipoproteins are also passively transferred onto red cells, red cells of secretors have ABH antigens not only on Type 2 precursor chains, but also on small numbers of Type 1 chains. In contrast, red cells of non-secretors have ABH antigens only on Type 2 chains.

Initially, it was thought that the *H* gene was a structural gene coding for the H enzyme and the secretor locus encoded a regulatory gene that permitted expression of the *H* gene in the relevant tissues. This hypothesis suggested that a single H enzyme transferred fucose in an α1-2 linkage to the terminal galactose residue on either Type 1 or Type 2 chains. In this model, the H enzyme is always expressed in red cell precursors, but expression in “secretory” tissues (eg, salivary epithelium) is controlled by the *Se* gene. However, this model did not explain all the available data, and multiple biochemical, immunologic, and genetic studies supported an alternative model.<sup>67,68</sup> The latter postulated two different H transferases: one adding fucose to Type 1 chains (an H Type 1 enzyme) and one acting on Type 2 chains (an H Type 2 enzyme). In this model, the H Type 1 enzyme is the structural protein encoded by the secretor gene (*Se*) and is expressed only in secretory tissues.

### Se and H Genes

The cloning of genes and cDNAs encoding multiple mammalian fucosyltransferases (for review, see Oriol et al<sup>69</sup>) show that *Se* is

the *FUT2* gene and that the product of the *Se* gene, the H Type 1 enzyme, is the *Se* (or Fuc-TII) enzyme.<sup>12,70</sup> At least one copy of the *Se* gene is found in approximately 80% of individuals and leads to ABH antigen expression in secretions. Indeed, *FUT2* mRNA expression is found in both normal epithelial cells<sup>12</sup> and cell lines derived from carcinomas,<sup>71</sup> but not in cell lines derived from hematopoietic neoplasia.<sup>71</sup> By contrast, the traditional *H* locus encodes the H Type 2 enzyme; the latter is equivalent to the H (or Fuc-TI) fucosyltransferase.<sup>72</sup> This gene (*H* or *FUT1*) is active in virtually all individuals (for exceptions, see below) and leads to ABH antigen expression on red cells and other tissues. The *FUT1* (or *H*) and *FUT2* (or *Se*) genes are closely linked on chromosome 19 (Table 6-2).<sup>11</sup> In addition, a third gene in this region, *Sec1*, has been cloned.<sup>12</sup> *Sec1* is a pseudogene of *FUT2*<sup>12</sup> and, although actively transcribed in human cell lines,<sup>71</sup> but perhaps not in normal human tissues,<sup>12</sup> it has no intrinsic enzymatic activity. These genes are arranged on chromosome 19 in the following sequence: Centromere → *Sec1* → *FUT2* → *FUT1* → Telomere. In addition, the high homology of these three genes, and the proximity of many *Alu* sequences, make this region a hot spot for mutation and genetic recombination<sup>71</sup> (see below).

### The Se (Fuc-TII) Glycosyltransferase

The *Se* enzyme is a Type II membrane glycoprotein containing 332 amino acids, a short cytosolic domain, and a large luminal domain (see Figure 6-2).<sup>70</sup> There are three potential N-glycosylation sites in the luminal domain at N177, N271, and N297. Its overall structure is similar to that of other mammalian glycosyltransferases, including the A and B enzymes. By analyzing the sequences of many eukaryotic and prokaryotic (α1-2)fucosyltransferases, three highly conserved sequence motifs were identified.<sup>69</sup> Motifs I, II, and III encompass amino acid residues 184-204, 226-239, and 278-288, respectively; Motif I may be responsible for binding the nucleotide sugar substrate, GDP-fucose, required for the catalytic action of this enzyme.



**Table 6-7.** Functional Alleles of the *Secretor* (*Se* or *FUT2*) Gene: cDNA Sequences

|                         | Nucleotide Residues |        |       |        |        |        |        |        |        |        |
|-------------------------|---------------------|--------|-------|--------|--------|--------|--------|--------|--------|--------|
|                         | 40                  | 210    | 278   | 357    | 375    | 379    | 385    | 480    | 481    | 855    |
| <b>Alleles</b>          |                     |        |       |        |        |        |        |        |        |        |
| <i>Se</i>               | A                   | a      | C     | c      | a      | C      | A      | c      | G      | a      |
| <i>Se</i> <sup>1</sup>  |                     |        |       | t      |        |        |        |        |        |        |
| <i>Se</i> <sup>2</sup>  | G                   |        |       |        |        |        |        |        |        |        |
| <i>Se</i> <sup>3</sup>  |                     |        |       |        |        |        |        |        | A      |        |
| <i>Se</i> <sup>4</sup>  | G                   |        |       |        |        |        |        |        | A      |        |
| <i>Se</i> <sup>5</sup>  |                     |        |       |        | g      |        |        |        |        |        |
| <i>Se</i> <sup>6</sup>  |                     |        |       | t      |        |        |        | t      |        |        |
| <i>Se</i> <sup>7</sup>  |                     |        |       | t      |        | T      |        | t      |        |        |
| <i>Se</i> <sup>8</sup>  |                     | a      |       | t      |        |        |        |        |        |        |
| <i>Se</i> <sup>9</sup>  |                     |        |       | t      |        |        |        |        |        | c      |
| <i>Se</i> <sup>10</sup> |                     |        | T     | t      |        |        |        |        |        |        |
| <i>Se</i> <sup>w</sup>  |                     |        |       | t      |        |        | T      |        |        |        |
| <b>Mutation</b>         | 14I→V               | silent | 93A→V | silent | silent | 127R→C | 129I→F | silent | 161D→N | silent |

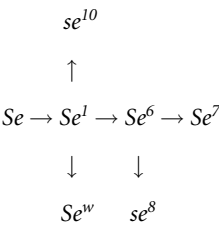
The H type 1 transferase (FUT2) is a 365 amino acid, Type II membrane glycoprotein. All sequences in this table are compared with a “parental” *Se* sequence. There is no generally agreed upon nomenclature for naming variant alleles of the *Se* gene; the system chosen here is based on the literature, but is otherwise arbitrary.

Following cloning of *Se*,<sup>12,70</sup> multiple functional and non-functional variants were described. For example, many individuals have a normally functioning variant, *Se*<sup>1</sup>, which contains a silent mutation: 357 c → t (Table 6-7).<sup>73-77</sup> In other individuals, normally functioning genes are found that contain only missense mutations (eg, *Se*<sup>2</sup>, *Se*<sup>3</sup>, *Se*<sup>4</sup>) or combinations of silent and missense mutations (eg, *Se*<sup>6</sup> and *Se*<sup>7</sup>).<sup>76,77</sup> In addition, the *Se*<sup>w</sup> mutant encodes a functional, but significantly less active, enzyme.<sup>74,78,79</sup> This allele contains a missense mutation that leads to partial expression of the secretor phenotype; individuals with this allele can have red cells with the unusual Le(a+b+) Lewis blood group phenotype (see below). None of the mutations found in functional *Se* variants occur in any of the three highly conserved (α1-2)fucosyltransferase motifs (Table 6-7).

In contrast, at least 10 nonenzymatically active variants of the *Se* gene have been described (Table 6-8). Most of these are caused by nonsense (ie, *se*<sup>1</sup>, *se*<sup>2</sup>, *se*<sup>3</sup>, *se*<sup>4</sup>, *se*<sup>5</sup>, *se*<sup>8</sup>) or small deletion (ie, *se*<sup>9</sup>) mutations that yield premature stop codons, resulting in the expression of truncated, nonfunctional enzyme proteins.<sup>70,73,75-77,80-82</sup> However, the *se*<sup>7</sup> allele contains one missense mutation at amino acid 101 that presumably encodes a full-length nonfunctional enzyme; nonetheless the effect of this mutation on enzyme function was not directly tested with recombinant protein.<sup>83</sup> Similarly, the *se*<sup>10</sup> allele contains a three base-pair deletion resulting in the deletion of a single amino acid, V230; although the encoded protein is presumably nonfunctional, this was not verified by transfection studies.<sup>84,85</sup> The mutation in the *se*<sup>10</sup> allele occurs in the highly conserved (α1-2)fucosyltransferase Motif II (Fig 6-5 and 6-7). One nonfunctional allele, *se*<sup>6</sup>, is the result of a fusion gene between *FUT2* (ie, *Se*) and the adjacent upstream *Sec1* pseudogene.<sup>75,80</sup> Although the recombinant *se*<sup>6</sup> protein has low, but measurable, enzymatic

activity using model substrates in vitro, it did not produce H antigen when expressed in transfected cells. Finally, the complete deletion of the entire *FUT2* gene is seen in individuals with the classical Bombay phenotype (see below); this allele is denoted as *se*<sup>del</sup>.<sup>86,87</sup> This large deletion is mediated by the presence of *Alu* sequences,<sup>88</sup> many of which are present in this chromosomal region.<sup>71</sup>

Given the abundance of sequencing data, it is possible to speculate on the temporal sequence during which the various mutations in the *Se* gene developed. One possible sequence is provided below:



This model supports the “out of Africa” hypothesis for the evolution of human populations. The *Se* and *Se*<sup>1</sup> genes are commonly found in Black, White, and Asian populations. In contrast, *se*<sup>10</sup> and *Se*<sup>w</sup> have been found only in Asian populations.<sup>74,78,79,84</sup> In addition, *Se*<sup>6</sup> has been found in Black and White, but not Asian, populations.<sup>76</sup> Finally, *se*<sup>8</sup> and *Se*<sup>7</sup> have been found only in Black and White populations, respectively.<sup>76</sup>

**The H (Fuc-TI) Glycosyltransferase**

The H enzyme contains 365 amino acids and is highly homologous to the *Se* enzyme (Fig 6-2).<sup>70</sup> In particular, it also contains the three highly conserved (α1-2)fucosyltransferase sequence motifs: Motifs I, II, and III comprise amino acids 214-224, 256-269, and 308-318,

**Table 6-8.** Nonfunctional Alleles of the *Secretor* (*Se* or *FUT2*) Gene: cDNA Sequences

|                  | Nucleotide Residues |        |        |        |           |        |           |           |           |         |        |           |           |        |
|------------------|---------------------|--------|--------|--------|-----------|--------|-----------|-----------|-----------|---------|--------|-----------|-----------|--------|
|                  | 171                 | 216    | 302    | 357    | 428       | 480    | 571       | 628       | 658       | 685     | 739    | 778       | 849       | 960    |
| Alleles          |                     |        |        |        |           |        |           |           |           |         |        |           |           |        |
| Se               | a                   | c      | C      | c      | G         | c      | C         | C         | C         | G       | G      | C         | G         | a      |
| se <sup>1</sup>  | g                   | t      |        |        | A         |        |           |           |           |         | S      |           |           | g      |
| se <sup>2</sup>  |                     |        |        |        |           |        | T         |           |           |         |        |           |           |        |
| se <sup>3</sup>  |                     |        |        | t      |           |        | T         |           |           |         |        |           |           |        |
| se <sup>4</sup>  |                     |        |        |        |           |        |           |           |           |         |        |           | A         |        |
| se <sup>5</sup>  |                     |        |        |        |           |        |           | T         |           |         |        |           |           |        |
| se <sup>6</sup>  |                     |        |        |        |           |        |           |           |           |         |        |           |           |        |
| se <sup>7</sup>  |                     |        | T      |        |           |        |           |           |           |         |        |           |           |        |
| se <sup>8</sup>  |                     |        |        | t      |           | t      |           |           |           |         |        | ΔC        |           |        |
| se <sup>9</sup>  |                     |        |        |        |           |        |           |           | T         |         |        |           |           |        |
| se <sup>10</sup> |                     |        |        | t      |           |        |           |           |           | ΔGTG    |        |           |           |        |
| Mutation         | silent              | silent | 101P→L | silent | 143W→stop | silent | 191R→stop | 210R→stop | 220R→stop | 230V→ΔV | 247G→S | 260P→stop | 283W→stop | silent |

All sequences in this table are compared with a “parental” *Se* sequence. There is no generally agreed upon nomenclature for naming variant alleles of the *Se* gene; the system chosen here is based on the literature, but is otherwise arbitrary. In the *se*<sup>1</sup> allele, the amino acid change at position 247 is downstream of the stop codon at position 143. The *se*<sup>6</sup> allele is a fusion gene that encodes an unusual chimeric protein where the N-terminus is encoded by the *Sec1* pseudogene, which is homologous to *Se*, and the C-terminus is encoded by the *Se* gene.<sup>75</sup>

In the *se*<sup>8</sup> allele, the frameshift mutation induced by the deletion at nucleotide 778 allows for the translation of a length of amino acid sequence that differs from the *Se* sequence before an early termination signal at a new stop codon. In the *se*<sup>10</sup> allele, the three nucleotide deletion (ie, GTG) at position 685 does not lead to a shift in the reading frame, but just leads to a deletion of a single amino acid (V230).

The *se*<sup>del</sup> allele, which is not included in this table, represents a complete deletion of the *Se* gene and is found in individuals with the Bombay phenotype.

respectively.<sup>69</sup> Based on the premise that the *H* gene codes for the H Type 2 glycosyltransferase (FUT1), then the allelic *h* gene codes for a nonfunctional enzyme. Since the cloning and sequencing of the *H* gene,<sup>72</sup> various groups have investigated the molecular nature of the defective *h* alleles.<sup>86,89-95</sup> More than 25 different *h* alleles have been identified, variously containing missense, nonsense, and frameshift mutations (Table 6-9). It is noteworthy that most families examined in these studies each had a different mutation leading to inactivation of Fuc-TI activity. In addition, several missense mutations occurred in the (α1-2)fucosyltransferase motifs; for example, the amino acid substitution 220 R → C occurs in Motif I, 259 V → E and 262 S → K occur in Motif II, and 315 A → V occurs in Motif III. The mutation at position 220 is particularly interesting in that the arginine at this position, in the putative nucleotide sugar binding domain, is absolutely conserved in (α1-2) and (α1-6)fucosyltransferases throughout evolution from bacteria to mammals.<sup>97</sup> Although the 327 N → T mutation does not occur in an (α1-2)fucosyltransferase motif, it does destroy a potential N-glycosylation site, suggesting that this posttranslational modification is important in the activity of this enzyme. In addition to the variants described above, one individual had a completely normal coding sequence, but no enzyme activity,<sup>92</sup> suggesting a defect in transcriptional regulation rather than in protein structure. This latter result shows that caution is required when molecular approaches for blood typing based on genotype are used, rather than classical methods, which directly determine blood group phenotype.

### H-Deficient Phenotypes and Genotypes

Based on the information described above, it should be clear that there are multiple molecular mechanisms by which an individual could express low amounts of H antigens on their red cells and/or in their secretions.

The most common example of an H-deficient individual is a “classical non-secretor,” that is, an individual with the general genotype *Hh sese* or *HH sese* (see Table 6-10). This individual has normal amounts of H Type 2 antigens on red cells, but does not express H Type 1 antigens in secretions. In this case, the individual would carry two *se* alleles, which could theoretically be combinations of any of the following: *se*<sup>1</sup>, *se*<sup>2</sup>, *se*<sup>3</sup>, *se*<sup>4</sup>, *se*<sup>5</sup>, *se*<sup>6</sup>, *se*<sup>7</sup>, *se*<sup>8</sup>, *se*<sup>9</sup>, *se*<sup>10</sup>, or *se*<sup>del</sup>.

“Classical weak secretors” are those individuals who are either homozygous or heterozygous for wild-type *H* and are either *Se<sup>w</sup>Se<sup>w</sup>* or *Se<sup>w</sup>se* at the secretor locus. *Se<sup>w</sup>* is a weak secretor allele (see above) that can synthesize small amounts of H Type 1 antigens and allows expression of the unusual Le(a+b+) phenotype (see below).

The most striking example of an H-deficient phenotype is the “classical Bombay phenotype,” often denoted as O<sub>h</sub>.<sup>98</sup> These individuals were originally identified in Bombay, India, their red cells type as group O, and they are non-secretors of ABH antigens. In addition, they do not express any H antigens on their red cells and their sera contain high-titered, IgM, hemolytic H antibodies that induce complement-mediated lysis of red cells of individuals

Table 6-9. *h* Alleles: Weak or Inactive Alleles of *FUT1*

| Nucleotide Change         | Amino Acid Change  | Enzymatic Activity | Ref.       |
|---------------------------|--------------------|--------------------|------------|
| <b>Missense Mutations</b> |                    |                    |            |
| 349 C → T                 | 117 H → Y          | Weak               | 86         |
| 442 G → T                 | 148 D → Y          | Weak               | 90, 96     |
| 460 T → C                 | 154 Y → H          | Weak               | 90, 93, 96 |
| 460 T → C                 | 154 Y → H          | Weak or absent     | 90, 93     |
| 1042 G → A                | 348 E → K          |                    |            |
| 461 A → G                 | 154 Y → C          | None               | 92         |
| 491 T → A                 | 164 L → H          | None               | 89         |
| 513 G → C                 | 171 W → C          | None               | 92         |
| 658 C → T                 | 220 R → C          | None               | 96         |
| 721 T → C                 | 241 Y → H          | Weak               | 90         |
| 725 T → G                 | 242 L → R          | None               | 86-88      |
| 776 T → A                 | 259 V → E          | None               | 92         |
| 785 G → A                 | 262 S → K          | None               | 94         |
| 786 C → A                 |                    |                    |            |
| 944 C → T                 | 315 A → V          | None               | 92         |
| 980 A → C                 | 327 N → T          | None               | 96         |
| 1042 G → A                | 348 E → K          | Weak               | 90, 93     |
| 1047 G → C                | 349 W → C          | None               | 92         |
| <b>Nonsense Mutations</b> |                    |                    |            |
| 538 C → T                 | 180 Q → stop       | None               | 95         |
| 1089 t → g                | silent             |                    |            |
| 547 ΔAG                   | 182 R → frameshift | None               | 96         |
| 695 G → A                 | 232 W → stop       | None               | 90         |
| 826 C → T                 | 276 S → stop       | None               | 89         |
| 880 ΔTT                   | 294 F → frameshift | None               | 96         |
| 948 C → G                 | 316 Y → stop       | None               | 89         |
| 969 ΔCT                   | 323 V → frameshift | None               | 92         |
| 990 ΔG                    | 330 L → frameshift | Weak               | 90         |

This table summarizes results published in peer-reviewed papers; several additional mutations have been reported in abstract form. The activity of each *h* allele was determined by transfection and expression of the recombinant protein and/or inferred on the basis of serologic studies of the red cells of the corresponding propositus. The *h* allele containing the 327 (N → T) amino acid mutation also contains an amino acid mutation at position 12 (A → V). However, because the latter is a conservative change in the putative transmembrane domain, it probably is not involved in the function of this enzyme.

The *h* allele containing the 180 (Q → stop) mutation in the amino acid sequence also contains a silent nucleotide change (1089 t → g), which does not change the amino acid code, and is downstream of the stop codon.

One *h* allele has been identified in which there are no mutations in the coding sequence; the lack of enzymatic activity is presumably due to a mutation(s) in a regulatory sequence.<sup>92</sup>

of any ABO group, except those from another individual with a similar type of global deficiency of H antigens. Individuals with the classical Bombay phenotype are homozygous for an *h* allele with the amino acid substitution 242 L → R; they are also homozygous for a deletion of the entire *FUT2* gene, that is, the *se<sup>del</sup>* allele of *Se*.<sup>86-88</sup> In this way, they are incapable of synthesizing either H Type 1 or H Type 2 structures. Although some of these individuals do express

active A and/or B enzymes, the A (or B) antigen is not detected because the appropriate substrate for these enzymes (ie, H Type 1 or H Type 2 chains) is not synthesized (Table 6-6). Nonetheless, the functional A (or B) genes can be transmitted to the next generation, yielding the apparently paradoxical pedigree O × O → A (or B).

On rare occasions, individuals are identified with ABH-deficient red cells but with ABH antigens in their secretions; this is often referred to as the “para-Bombay phenotype.”<sup>89-91,93,99</sup> However, the literature can be confusing on this point and this broad phenotypic description can, at least theoretically, encompass individuals with functional *Se* alleles and nonfunctional *H* alleles, or with weakly functioning *H* alleles and nonfunctional *Se* alleles (see Table 6-10). Thus, individuals with the “RBC H-deficient, secretor,” “RBC H-deficient, weak secretor,” and “RBC weak H, non-secretor” phenotypes could all be described as having the para-Bombay phenotype. Therefore, confusion can be avoided by using more specific terms that describe the types of alleles an individual has at the *Se* and *H* loci (Table 6-10).

Medical Implications of the ABH and Secretor Systems

In transfusion medicine, the major implications of these systems relate to the occurrence of serious, acute hemolytic transfusion reactions in patients receiving incompatible red cells (eg, red cells from a group A donor transfused to a group O recipient, or red cells from a regular donor transfused to a Bombay O recipient). The incidence, pathophysiology, prevention, and treatment of such reactions are discussed elsewhere in this book. However, these blood group systems are also relevant in several other situations, examples of which are described below.

Biological Relevance of the ABH Antigens in Coagulation

The normal physiologic functions of the ABO histo-blood group system are still unknown. However, the level of expression of the A/B antigens on the N-glycans of the von Willebrand factor (vWF) procoagulant glycoprotein affects circulating plasma levels of both vWF and Factor VIII, the latter of which is protected from degradation by its association with vWF.<sup>100</sup> Thus, group AB individuals have the highest levels of vWF followed by those of group B, A, and then O, who have the lowest. This finding correlates well with the increased incidence of thrombotic diseases in non-group-O individuals as opposed to the increased bleeding tendency in group O individuals. Nonetheless, the precise mechanisms underlying how the presence of terminal ABH structures on vWF N-glycans may affect overall thrombotic risk are still unclear,<sup>101</sup> although their contribution to the resistance of vWF to degradation by ADAMTS13 may be critically important.<sup>102</sup>

The Role of the ABH Antigens in Malaria: A New Hypothesis

A fascinating new hypothesis may provide a comprehensive explanation of the distribution of the ABO blood groups in various human populations.<sup>103</sup> This unifying hypothesis may also explain variations in the Secretor system and the Lewis blood group system (see below). In brief, this hypothesis addresses the pathophysiology of adherence of red cells infected with

**Table 6-10.** Phenotypes and Genotypes Involving *FUT1* and *FUT2*

| Phenotype                   | RBC ABH<br>Antigen Level | Salivary ABH<br>Antigen Level | <i>H (FUT1)</i> Gene      | <i>Se (FUT2)</i> Gene                                          |
|-----------------------------|--------------------------|-------------------------------|---------------------------|----------------------------------------------------------------|
| Classical secretor          | Normal                   | Normal                        | <i>HH</i> or <i>Hh</i>    | <i>SeSe</i> or <i>Sese</i>                                     |
| Classical non-secretor      | Normal                   | Absent                        | <i>HH</i> or <i>Hh</i>    | <i>sese</i>                                                    |
| Classical weak secretor     | Normal                   | Weak                          | <i>HH</i> or <i>Hh</i>    | <i>Se<sup>w</sup>Se<sup>w</sup></i> or <i>Se<sup>w</sup>se</i> |
| Classical Reunion phenotype | Weak                     | Absent                        | <i>hh</i> : 117 H → Y     | <i>se<sup>l</sup>se<sup>l</sup></i>                            |
| RBC weak H, non-secretor    | Weak                     | Absent                        | <i>hh</i> : eg, 241 Y → H | <i>sese</i>                                                    |
| RBC weak H, weak secretor   | Weak                     | Weak                          | <i>hh</i> : eg, 241 Y → H | <i>Se<sup>w</sup>Se<sup>w</sup></i> or <i>Se<sup>w</sup>se</i> |
| RBC weak H, secretor        | Weak                     | Normal                        | <i>hh</i> : eg, 241 Y → H | <i>SeSe</i> or <i>Sese</i>                                     |
| Classical Bombay phenotype  | Absent                   | Absent                        | <i>hh</i> : 242 L → R     | <i>se<sup>del</sup>se<sup>del</sup></i>                        |
| Other "Bombay" phenotype    | Absent                   | Absent                        | <i>hh</i>                 | <i>sese</i>                                                    |
| H-deficient, weak secretor  | Absent                   | Weak                          | <i>hh</i>                 | <i>Se<sup>w</sup>Se<sup>w</sup></i> or <i>Se<sup>w</sup>se</i> |
| H-deficient, secretor       | Absent                   | Normal                        | <i>hh</i>                 | <i>SeSe</i> or <i>Sese</i>                                     |

Where the type of *Se*, *se*, or *h* allele is not specified, various alleles described in Tables 6-7 through 6-9 could theoretically be used. Most, if not all, of the phenotypes in this table have been described in the literature using serologic and/or genetic methods. The "RBC H-deficient, weak secretor" and the "RBC H-deficient, secretor" phenotypes are often described as para-Bombay phenotypes; however, on occasion, the "RBC weak H, non-secretor" phenotype can also be described as a "para-Bombay" phenotype, which can lead to confusion.

*Plasmodium falciparum* to capillary endothelium, which is particularly relevant for the severe, and often fatal, complication of cerebral malaria. Thus, the surface of infected red cells expresses the malarial PfEMP-1 protein, which has lectin-like properties with specificity for the A (and B) oligosaccharide structures. In this way, infected red cells could adhere directly to endothelial cells bearing A (or B) antigens. Alternatively, infected red cells could indirectly adhere to endothelial cells by first binding to either platelets or vWF expressing A (or B) structures. If correct, this hypothesis would predict that individuals who did not express A or B antigens (ie, group O individuals) would be at least partially protected from the severe consequences of *P. falciparum* malaria. Similarly, the presence of the Secretor phenotype would be somewhat protective in group A or B individuals, because the A/B antigens on plasma lipoproteins would block endothelial adherence of infected red cells. This protection would be enhanced if these particular individuals also lacked Lewis antigens (see below); that is, if they had the Le(a-b-) phenotype. In summary, this hypothesis would explain the relative abundance of the group O and Le(a-b-) phenotypes in populations that have a long history of exposure to *P. falciparum* malaria. It will be of great interest to see whether future studies confirm this novel hypothesis.

### ABO-Incompatible Solid Organ Transplantation

The increasing number of patients needing organ transplantation and the shortage of available organs led to studies of the feasibility of ABO-incompatible (ABOI) transplantation. Because of naturally occurring IgM and IgG anti-A/B found in "unimmunized" naïve individuals, early attempts at ABOI transplantation resulted in hyperacute or acute rejection.<sup>104,105</sup> However, protocols allowing for successful ABOI transplantation were subsequently developed. These typically involve removal of anti-donor,

anti-A/B by plasmapheresis before transplantation along with pharmacologic immunosuppressive agents, often combined with splenectomy.<sup>106,107</sup> Using these approaches, the overall 1-year graft survival is approximately 50% to 60% following ABOI kidney, liver, or heart transplantation, compared with 70% to 80% for ABO-compatible transplantation.<sup>108</sup>

ABOI kidney transplants in adults have been performed in significant numbers and with quite good success, especially in Japan where religious beliefs preclude the use of cadaveric organs.<sup>109</sup> In particular, the reduced antigen expression in blood group A<sub>2</sub> donors results in protection from antibody-mediated injury; thus, group A<sub>2</sub> kidneys can be transplanted into group B or O individuals without prior depletion of anti-A.<sup>110</sup>

ABOI liver transplants in adults tend to have a poor outcome because of major complications associated with high preoperative IgM and IgG anti-A/B titers.<sup>111</sup> Graft survival of ABOI liver transplants is still substantially lower than for ABO-matched transplants; therefore, ABOI liver transplants are reserved for emergent situations when a compatible donor cannot be found.<sup>112</sup> In addition, in ABO-compatible but nonidentical transplants (ie, "minor" mismatch), graft-derived anti-A/B can be produced by passenger lymphocytes, leading to often mild, but occasionally serious, hemolysis of the recipient's red cells.<sup>113</sup>

ABOI heart transplants in adults are associated with high mortality.<sup>114</sup> In contrast, following preoperative antibody reduction, ABOI heart transplants in newborns and infants have been relatively successful, especially considering the high mortality associated with extended stays on the transplant waiting list.<sup>115</sup>

### ABOI Hematopoietic Stem Cell Transplantation

Because of the distribution of ABO blood types in human populations, many allogeneic hematopoietic stem cell transplants are ABO incompatible.<sup>108</sup> These may result from a major incompatibility

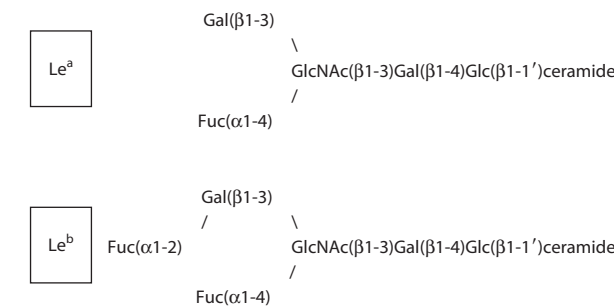


**Figure 6-4.** Biochemical structures of the Lewis-active glycosphingolipids. Glc = D-glucose; GlcNAc = D-N-acetylglucosamine; cer = ceramide; Gal = D-galactose; Fuc = fucose.

(eg, group A or B donor cells transplanted into a group O recipient),<sup>116</sup> where the primary immediate risk from the procedure is the transfusion of ABO-incompatible red cells present in the hematopoietic stem cell product. This type of risk can be ameliorated by removing red cells in the donor product and/or removing anti-A/B in the recipient by plasmapheresis. In contrast, transplants involving a minor ABO mismatch (eg, group O donor cells transplanted into a group A or B recipient) may produce severe delayed hemolysis resulting from anti-A/B production by passenger lymphocytes in the graft;<sup>117</sup> various methods can be employed to address this issue (for example, see Worel et al<sup>118</sup>).

The Lewis System

The two Lewis blood group antigens Le<sup>a</sup> (Lewis a) and Le<sup>b</sup> (Lewis b) were discovered in the 1940s (for review, see Henry et al<sup>119</sup>). Virtually all individuals fall into one of three different Lewis types: Le(a+b−), Le(a−b+), and Le(a−b−) (Table 6-11). These molecules are not intrinsic red cell antigens; they are synthesized in another tissue (probably intestinal epithelium), circulate in plasma attached to lipoproteins, and then passively transfer onto red cells.<sup>120</sup> Biochemically these are carbohydrate antigens on glycosphingolipids (Fig 6-4). They are structurally similar to the Type 1 ABH antigens found on plasma glycosphingolipids, which can also transfer onto red cells.

The Lewis gene (*Le*) is on chromosome 19<sup>11,14</sup> and is distantly linked to the *H* and *Se* loci (Table 6-2). The gene encodes an (α1-4)fucosyltransferase, denoted Fuc-TIII, and thus behaves in a dominant fashion. A human cDNA derived from the *Le* gene (equivalently, the *FUT3* gene) was cloned and encodes a 361 amino acid Type II membrane-bound glycoprotein (Fig 6-2).<sup>121</sup> In addition to adding fucose in an α1-4 linkage to the GlcNAc residue on a Type 1 chain, it can also add fucose in an α1-3 linkage to the GlcNAc on a Type 2 chain (Table 6-6). Although the latter reaction is relatively inefficient, it forms the Lewis x antigen, Le<sup>x</sup>, an important tumor-associated antigen. Thus, Fuc-TIII is a very unusual glycosyltransferase in that it can transfer a monosaccharide to two very different substrates using different glycosidic linkages; therefore, it is technically an (α1-3/4)fucosyltransferase.<sup>121</sup> The sequence near its NH2-terminus is important

**Table 6-11.** The Lewis Blood Group System

| Blood Group | Red Cell Antigens |                 | Serum Antibodies     |                      |
|-------------|-------------------|-----------------|----------------------|----------------------|
|             | Le <sup>a</sup>   | Le <sup>b</sup> | Anti-Le <sup>a</sup> | Anti-Le <sup>b</sup> |
| Le(a+b−)    | +                 | −               | —                    | Very Rarely          |
| Le(a−b+)    | −                 | +               | Very Rarely          | —                    |
| Le(a−b−)    | −                 | −               | Occasionally         | Occasionally         |

for determining its acceptor specificity.<sup>122-126</sup> Thus, mutating the sequence of Fuc-TIII at residues 111 and 112 to that which is found in the (α1-3)fucosyltransferase Fuc-TVI (ie, from WD in Fuc-TIII to RE in Fuc-TVI), significantly improves its ability to use Type 2 chains as substrates.<sup>122</sup> By sequence analysis, Fuc-TIII is a member of a family of (α1-3)fucosyltransferases that is conserved from bacteria to mammals.<sup>69,97</sup> As such, there are two highly conserved sequence motifs: Motif I comprising amino acids 152-171 and Motif II comprising amino acids 239-272. Fuc-TIII also contains two potential N-glycosylation sites; the site at N154 is in sequence Motif I. Although FUT-TIII has not yet been crystallized, molecular modeling has provided some insight into its three-dimensional structure.<sup>127</sup>

The transfer of fucose to a Type 1 chain by Fuc-TIII results in formation of the Le<sup>a</sup> antigen; the addition of α1-4 linked fucose to the H Type 1 structure leads to formation of Le<sup>b</sup>. Thus, the latter is formed by the cooperation of two glycosyltransferases encoded by two genes, one from the Lewis system (*Le* on chromosome 19) and one from the ABH system (*Se* at a different locus on chromosome 19). The biosynthetic pathways connecting the ABH, Secretor, and Lewis systems (encoded by genes at three distinct loci on chromosomes 9 and 19) are shown in Fig 6-5. Cooperation of the *Le*, *Se*, and *A* (or *B*) genes leads to the formation of a minor antigen, AL<sup>b</sup> (or BL<sup>b</sup>), recognized by both anti-A (or anti-B) and anti-Le<sup>b</sup>. Because the *Se* enzyme (ie, Fuc-TII) converts virtually all Type 1 chains into H Type 1, whether or not Fuc-TIII is present, Lewis-positive secretors have virtually no Le<sup>a</sup> antigen and their red cells type as Le(a−b+). By contrast, Lewis-positive non-secretors have Le(a+b−) red cells. This is summarized in Table 6-11.

Individuals whose red cells type as Le(a+b+) are very unusual among Whites, but are relatively common in Asian populations. In these individuals the *Le* gene is normal but the *Se* (Fuc-TII) enzyme is partially defective due to a variant allele (*Se<sup>w</sup>*) containing the 129 I → F mutation<sup>74,77-79</sup> (Table 6-7). Because the *Se<sup>w</sup>* enzyme has partial activity, some Type 1 chains are converted to H Type 1, which are then converted to Le<sup>b</sup> by the normal Lewis enzyme, Fuc-TIII (Fig 6-5). In addition, some Type 1 chains avoid α1-2 fucosylation by the partially defective *Se<sup>w</sup>* enzyme and are converted into Le<sup>a</sup> by the normal Lewis Fuc-TIII enzyme (Fig 6-5). Thus, the red cells of these individuals contain both the Le<sup>a</sup> and Le<sup>b</sup> antigens and type as Le(a+b+).

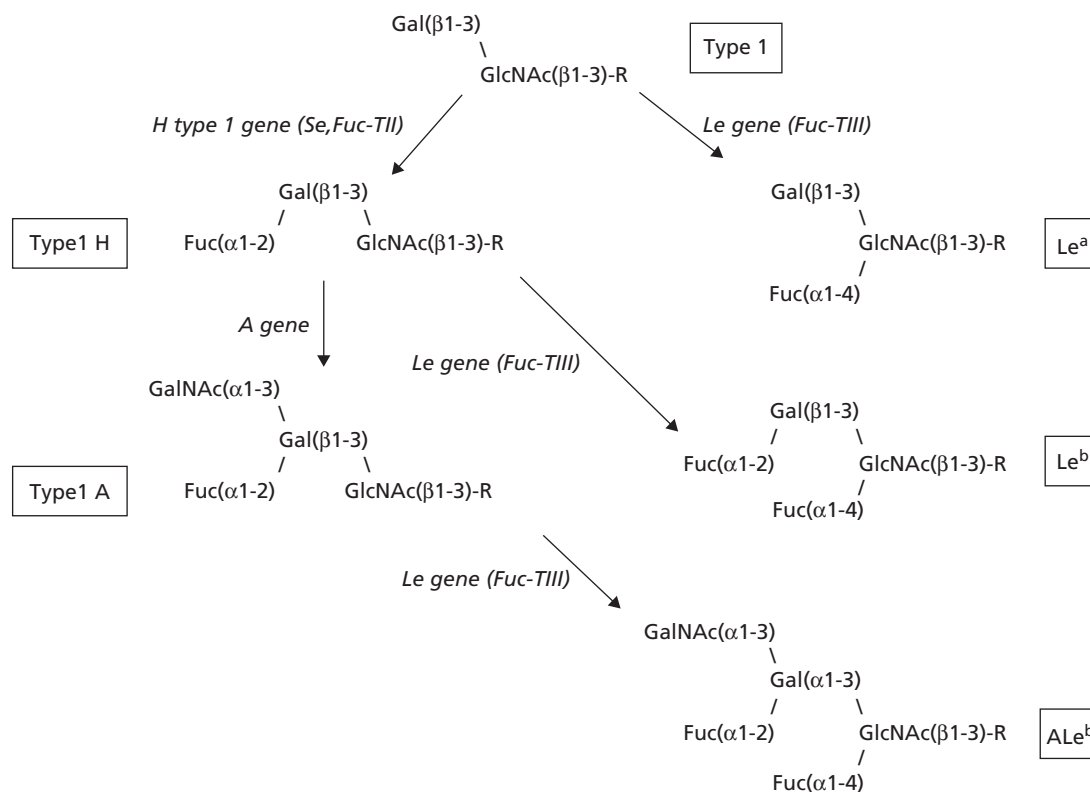


**Figure 6-5.** Biosynthesis of blood group antigens with Type 1 chains. The genes encoding the relevant glycosyltransferases are denoted in italics and the reactions catalyzed are indicated by arrows. The names of the individual blood group antigens are shown in boxes next to the relevant structures. GlcNAc = D-N-acetylglucosamine; Gal = D-galactose; GalNAc = D-N-acetylgalactosamine; Fuc = fucose.

The presence of defective *Le* alleles is relatively common and approximately 5% of Whites and 25% of Blacks type as *Le(a-b-)*. The increased prevalence of the *Le(a-b-)* phenotype in Blacks may have evolved from selective pressure due to infection with *P. falciparum* malaria (see above).<sup>103</sup> To date, at least 10 different defective *Le* alleles (*le<sup>1</sup>* through *le<sup>10</sup>*) have been identified (Table 6-12).<sup>128-135</sup> Based both on family studies and expression of transfected chimeric cDNAs in vitro, the mutations at amino acids 68,<sup>134</sup> 170,<sup>129,130,132</sup> 223,<sup>136</sup> 270,<sup>136</sup> and 356<sup>129-131</sup> severely inhibit or abolish enzyme activity. One of these mutations occurs in ( $\alpha 1-3$ )fucosyltransferase Motif I and one in Motif II. The mutation at amino acid 20, which is in the transmembrane domain, affects Golgi localization of this enzyme and its activity with glycosphingolipid and glycoprotein substrates, thus yielding a paradoxical “nongenuine” Lewis-negative phenotype.<sup>129-132</sup> That is, although red cells of individuals homozygous for *le<sup>3</sup>* lack Lewis-active glycosphingolipids and type as *Le(a-b-)*, their salivary mucins do contain Lewis antigens.<sup>131,137</sup>

Although hemolytic transfusion reactions and hemolytic disease of the fetus and newborn are rarely caused by antibodies to the Lewis blood group antigens, this blood group system may be important in renal transplantation,<sup>138</sup> coronary heart

disease,<sup>139,140</sup> gastrointestinal cancer,<sup>141</sup> infection by *Helicobacter pylori*,<sup>142,143</sup> and *P. falciparum* malaria (see above).<sup>103</sup>

## Ii Blood Group System

The Ii antigens are oligosaccharides that form the Type 2 chain precursors for the ABH antigens (Fig 6-6; see also Table 6-6). The best available evidence indicates that the difference between the I and i antigens relates to branching of the oligosaccharide chain; i antibodies recognize an unbranched oligosaccharide chain, and I antibodies recognize a similar chain that is also branched. Fetal and cord red cells contain mostly i antigen with small amounts of I; adult red cells demonstrate the opposite pattern. This suggests that the glycosyltransferase necessary for synthesis of the branched structure, a ( $\beta 1-6$ )N-acetylglucosaminyltransferase, is developmentally regulated. The availability of the cloned cDNA encoding both this enzyme<sup>16</sup> and the “i-extension” enzyme<sup>144</sup> (Table 6-2) allowed this hypothesis to be tested. Rare adults have red cells that type as i; these individuals have mutations in a particular isoform of ( $\beta 1-6$ )N-acetylglucosaminyltransferase and their red cell precursors lack the relevant branching glycosyltransferase activity.<sup>145-147</sup>

Table 6-12. Functional and Nonfunctional Alleles of the *Lewis* (*Le* or *FUT3*) Gene: cDNA Sequences

|                         | Nucleotide Sequence |       |        |        |        |        |        |        |        |        |
|-------------------------|---------------------|-------|--------|--------|--------|--------|--------|--------|--------|--------|
|                         | 59                  | 202   | 304    | 314    | 370    | 484    | 508    | 667    | 808    | 1067   |
| Alleles                 |                     |       |        |        |        |        |        |        |        |        |
| <i>Le</i>               | T                   | T     | C      | C      | T      | G      | G      | G      | G      | T      |
| <i>Le</i> <sup>1</sup>  |                     |       | A      |        |        |        |        |        |        |        |
| <i>Le</i> <sup>2</sup>  |                     |       |        |        | G      |        |        |        |        |        |
| <i>le</i> <sup>1</sup>  | G                   |       |        |        |        |        | A      |        |        |        |
| <i>le</i> <sup>2</sup>  | G                   |       |        |        |        |        |        |        |        | A      |
| <i>le</i> <sup>3</sup>  | G                   |       |        |        |        |        |        |        |        |        |
| <i>le</i> <sup>4</sup>  |                     |       |        |        |        |        |        |        |        | A      |
| <i>le</i> <sup>5</sup>  | G                   | C     |        |        |        |        |        |        |        | A      |
| <i>le</i> <sup>6</sup>  |                     | C     |        | T      |        |        |        |        |        |        |
| <i>le</i> <sup>7</sup>  |                     | C     |        |        |        |        |        |        |        |        |
| <i>le</i> <sup>8</sup>  |                     | C     |        | T      |        | A      |        |        |        |        |
| <i>le</i> <sup>9</sup>  |                     |       |        |        |        | A      |        | A      |        |        |
| <i>le</i> <sup>10</sup> |                     |       |        |        |        | A      |        | A      | A      |        |
| Mutation                | 20L→R               | 68W→R | 102Q→K | 105T→M | 124S→A | 162D→N | 170G→S | 223G→R | 270V→M | 356I→K |

The Lewis transferase (FUT3) is a 361 amino acid, Type II membrane glycoprotein. All sequences in this table are compared with a “parental” *Le* sequence. There is no generally agreed upon nomenclature for naming variant alleles of the *Le* gene; the system chosen here is based on the literature, but is otherwise arbitrary.

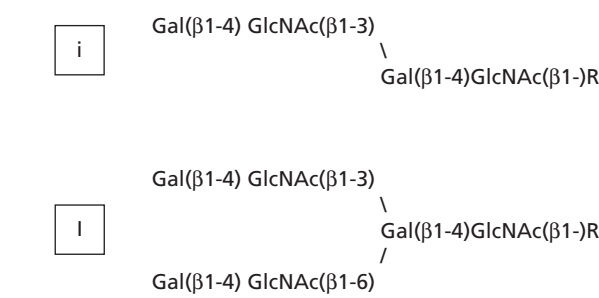


Figure 6-6. Ii blood group antigens. GlcNAc = D-N-acetylglucosamine; Gal = D-galactose; GalNAc = D-N-acetylgalactosamine.

Antibodies specific for the Ii antigens are clinically relevant in the setting of cold type autoimmune hemolytic anemia. The sera of these patients typically contain high titers of a monoclonal antibody, usually with anti-I specificity. In patients demonstrating hemolysis in vivo, the antibodies bind to red cells at or near 37°C in vitro and have the ability to fix complement. Surprisingly, almost all normal individuals have low titers of anti-I; these autoantibodies agglutinate red cells only at room temperature or below and do not cause accelerated red cell destruction in vivo. Patients with particular infectious diseases such as infectious mononucleosis and mycoplasmal pneumonia often develop cold agglutinins, typically with anti-i and anti-I specificity, respectively.<sup>148</sup> In unusual cases, this may result in immune-mediated hemolytic anemia. At present, there is not a complete understanding of the mechanisms

underlying the differences between cold agglutinins that cause hemolysis in vivo and those that do not.<sup>149-152</sup>

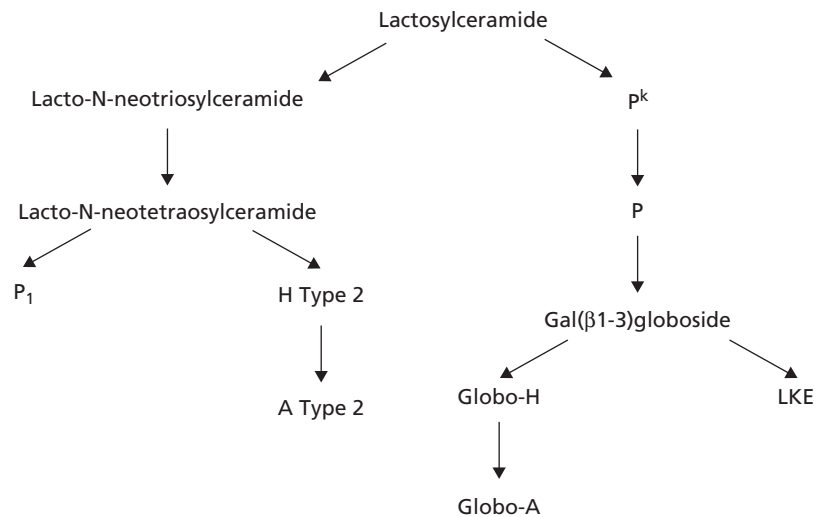
P Blood Group System

The P blood group system consists of at least three well-defined glycosphingolipid antigens (P<sup>k</sup>, P, and P<sub>1</sub>) and associated structures such as LKE, Globo-H, and Globo-A (Table 6-13) (for review, see Spitalnik<sup>133</sup>). These carbohydrate chains are related structurally and biosynthetically.<sup>154</sup> Each has a common precursor, Gal(β1-4)Glc-ceramide (also called lactosylceramide). The P<sup>k</sup> antigen is the biosynthetic precursor of the P antigen and both P<sup>k</sup> and P<sub>1</sub> share a common disaccharide structure, Gal(α1-4)Gal(β1-4)-R, at the nonreducing end of the glycosphingolipid. These antigenic structures are not found on red cell membrane glycoproteins.<sup>155</sup> The glycosyltransferase that converts lactosylceramide to the P<sup>k</sup> antigen is denoted as P<sup>k</sup> synthase, that which converts P<sup>k</sup> to P is denoted as P synthase, and that which converts paragloboside to P<sub>1</sub> is denoted as P<sub>1</sub> synthase (Tables 6-13 and 6-14, Fig 6-7). Although the human P<sup>k</sup> synthase was cloned,<sup>17,20</sup> the gene encoding the P<sub>1</sub> synthase has been localized to chromosome 22, but has not yet been cloned (Table 6-2).<sup>18,19</sup> Indeed, identifying the P<sub>1</sub> synthase gene has been quite controversial.<sup>156</sup>

Five different red cell phenotypes have been described in which various combinations of these three antigens are expressed (Table 6-14). The P<sub>1</sub> and P<sub>2</sub> phenotypes are common and account for almost the entire population. The serum of some P<sub>2</sub> individuals contains anti-P<sub>1</sub>. These antibodies are usually low-titered IgM cold

**Table 6-13.** Structures of P Blood Group and Related Glycosphingolipid Antigens

|                              |                                                               |
|------------------------------|---------------------------------------------------------------|
| Lactosylceramide             | Galβ1-4Glcβ1-1'cer                                            |
| P <sup>k</sup>               | Galα1-4Galβ1-4Glcβ1-1'cer                                     |
| P                            | GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1'cer                           |
| Gal-globoside                | Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1'cer                    |
| LKE                          | NeuAcα2-3Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1'cer           |
| Globo-H                      | Fucα1-2Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1'cer             |
| Globo-A                      | GalNAcα1-3[Fucα1-2]Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1'cer |
| Lacto-N-neotriaosylceramide  | GlcNAcβ1-3Galβ1-4Glcβ1-1'cer                                  |
| Lacto-N-neotetraosylceramide | Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1'cer                           |
| P <sub>1</sub>               | Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1'cer                    |
| H Type 2                     | Fucα1-2Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1'cer                    |
| A Type 2                     | GalNAcα1-3[Fucα1-2]Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1'cer        |

**Figure 6-7.** Biosynthetic pathway of antigens in the P blood group system and related structures. The biochemical structures of these glycosphingolipids are shown in Table 6-13.**Table 6-14.** Red Cell Phenotypes in the P Blood Group System

| Phenotype                   | Frequency | Red Cell Antigens                  | Enzymes Present                              | Serum Antibodies                                             |
|-----------------------------|-----------|------------------------------------|----------------------------------------------|--------------------------------------------------------------|
| P <sub>1</sub>              | 75%       | P <sub>1</sub> , P, P <sup>k</sup> | P <sub>1</sub> , P, P <sup>k</sup> synthases | None                                                         |
| P <sub>2</sub>              | 25%       | P, P <sup>k</sup>                  | P, P <sup>k</sup> synthases                  | Anti- P <sub>1</sub>                                         |
| P <sub>1</sub> <sup>k</sup> | Rare      | P <sub>1</sub> , P <sup>k</sup>    | P <sub>1</sub> , P <sup>k</sup> synthases    | Anti-P                                                       |
| P <sub>2</sub> <sup>k</sup> | Rare      | P <sup>k</sup>                     | P <sup>k</sup> synthase                      | Anti- P <sub>1</sub> , anti-P                                |
| p                           | Rare      | None                               | None                                         | Anti- P <sub>1</sub> PP <sup>k</sup> (anti-Tj <sup>a</sup> ) |

agglutinins and are rarely of clinical significance; however, rare acute hemolytic transfusion reactions have been described.<sup>157</sup> In contrast, the unusual individuals with the P<sub>1</sub><sup>k</sup>, P<sub>2</sub><sup>k</sup>, and p phenotypes<sup>158</sup> have naturally occurring high-titered IgM antibodies with specificity either for the P antigen (ie, anti-P) or for all the antigens in the P blood group system (ie, anti-P<sub>1</sub>PP<sup>k</sup>, or, equivalently, anti-Tj<sup>a</sup>). These antibodies are clinically relevant in that they can cause severe hemolytic transfusion reactions. An unusual syndrome of recurrent spontaneous abortions has also been associated with these antibodies,<sup>159</sup> presumably caused by the presence of P<sup>k</sup>- and P-active glycosphingolipids on trophoblastic tissue.<sup>160</sup> In addition, the syndrome

of paroxysmal cold hemoglobinuria, originally associated with syphilis, is caused by Donath-Landsteiner antibodies. The latter are cold-reacting, complement-fixing IgG antibodies with anti-P specificity that cause immune-mediated hemolysis in vivo.<sup>161</sup>

The P blood group antigens are present on multiple cell types in addition to red cells and have various functions. For example, the P antigen is the receptor for parvovirus B19 on erythropoietic precursors.<sup>162,163</sup> This virus causes both transient aplastic crises in patients with underlying hemolysis (eg, in sickle cell disease) and anemia in immunocompromised patients.<sup>164</sup> In addition, the P<sup>k</sup> antigen, also denoted as CD77, is the receptor on endothelial cells for *E. coli* verotoxins, which are important in the pathogenesis of the hemolytic uremic syndrome.<sup>165</sup> The LKE antigen also functions as a receptor for uropathogenic *E. coli*.<sup>166</sup> Finally, the P<sup>k</sup> antigen appears to be important in signal transduction following binding of α-interferon and also in apoptosis.<sup>167,168</sup>

## Summary

The carbohydrate blood group antigens that are most relevant for the practice of transfusion medicine are found in the ABH, Secretor,



Lewis, Ii, and P human blood group systems. These may be more appropriately regarded as “histo-blood group antigens” in that their expression is not restricted to red cells. The oligosaccharide structures in these systems are related to each other biochemically and immunologically. Great progress has been made in understanding the enzymatic pathways involved in synthesizing these antigens and in the molecular biology of the genes encoding these glycosyltransferases. Although the normal biological functions of these structures are not yet fully understood, many of them play important roles in human infectious diseases.

## Disclaimer

The authors have disclosed no conflicts of interest.

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# Rh and LW Blood Group Antigens

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Although it is now clear that the Rh and LW antigens are carried on entirely different proteins, they are incorporated together in this chapter on the basis of a historic serologic connection (and confusion) and, more recently, evidence that they are physically associated within the red cell membrane.

## Rh Blood Group System

### History and Nomenclature

The Rh system is second only to the ABO system in importance in transfusion medicine because Rh antigens, especially D, are highly immunogenic and cause hemolytic disease of the fetus and newborn (HDFN) and hemolytic transfusion reactions (HTRs). HDFN was first described by a French midwife in 1609 in a set of twins, one of whom was hydropic and stillborn, while the other was jaundiced and died of kernicterus.<sup>1</sup> That a wide range of observed clinical scenarios involving red cell hemolysis were related—from severely hydropic stillborn fetuses to infants with mild or significant levels of jaundice and kernicterus—was not realized until 1932.<sup>2</sup> The cause of red cell hemolysis remained elusive until 1939, when Levine and Stetson described a woman who delivered a stillborn fetus and also suffered a severe hemolytic reaction when transfused with blood from her husband. Levine and Stetson correctly surmised that the mother had been immunized by a fetal red cell antigen inherited from the father and suggested that the cause of the erythroblastosis fetalis was maternal antibody in the fetal circulation.<sup>3</sup> They did not give the target blood group antigen a name. Meanwhile Landsteiner and Wiener, in an effort to discover new blood groups, injected rabbits and guinea pigs with rhesus monkey red cells. The antiserum they obtained agglutinated not only rhesus monkey red cells, but also the red cells of 85% of White subjects, whom they called “Rh positive”; the remaining 15% of individuals studied were termed “Rh negative.”<sup>4(p195)</sup> The “anti-Rhesus” serum seemed to be

reacting similarly to the maternal antibody in serologic testing, hence the blood group system responsible for HDFN came to be known as “Rh.” It is now clear that the anti-Rhesus serum was detecting the LW antigen (subsequently named for Landsteiner and Wiener), which is present in greater amounts on D-positive than on D-negative red cells.<sup>4(p407)</sup> Years of debate followed concerning whether the human antibodies and the Rhesus antibodies were detecting the same antigen and extended long after serologic profiles suggested they were reacting with different structures. Landsteiner and Wiener never accepted the LW terminology, because doing so would imply that they had not discovered the cause of HDFN.<sup>5</sup>

It was soon obvious that Rh was not a simple, single-antigen system. In 1941, Fisher named the C and c antigens (A and B had been used for ABO), and used the next letters of the alphabet, D and E, to define antigens recognized by additional antibodies. In 1945, the e antigen was identified.<sup>4(p195)</sup>

It is important for transfusion medicine specialists to appreciate that the often confusing nomenclature used to describe Rh antigens results from the difference in opinion that existed concerning the number of genes that were involved in their expression. The Fisher-Race nomenclature suggested that three closely linked genes (C/c, E/e, and D) were responsible, while the Wiener nomenclature (Rh-Hr) was based on his belief that a single gene encoded one “agglutinin” that carried several blood group factors. Even though neither theory was correct (there are two genes—*RHD* and *RHCE*—correctly proposed by Tippett<sup>6</sup>), for written communication the Fisher-Race designation (CDE) for haplotypes is preferred, and for spoken communication a modified version of Wiener’s nomenclature is preferred (Table 7-1). The “R” indicates that D is present and use of a lowercase “r” (or “little r”) indicates that it is not. The C or c and E or e Rh antigens carried with D are represented by subscripts: 1 for Ce (R<sub>1</sub>), 2 for cE (R<sub>2</sub>), 0 for ce (R<sub>0</sub>), and Z for CE (R<sub>Z</sub>). The CcEe antigens present without D are represented by superscript symbols: “prime” for Ce (r’), “double-prime” for cE (r’'), and “y” for CE (r<sup>y</sup>). The “R” versus “r” terminology allows one to convey the common Rh antigens present on one chromosomal haplotype in a single term (a phenotype).

Table 7-1. Nomenclature and Prevalence of Rh Haplotypes

| Haplotype-Based Antigens (Fisher/Race) | Shorthand for Haplotype (Modified Wiener) | Occurrence (%) |        |        |
|----------------------------------------|-------------------------------------------|----------------|--------|--------|
|                                        |                                           | Whites         | Blacks | Asians |
| D <sub>Ce</sub>                        | R <sub>1</sub>                            | 42             | 17     | 70     |
| D <sub>cE</sub>                        | R <sub>2</sub>                            | 14             | 11     | 21     |
| D <sub>ce</sub>                        | R <sub>0</sub>                            | 4              | 44     | 3      |
| D <sub>CE</sub>                        | R <sub>Z</sub>                            | <0.01          | <0.01  | 1      |
| ce                                     | r                                         | 37             | 26     | 3      |
| Ce                                     | r'                                        | 2              | 2      | 2      |
| cE                                     | r''                                       | 1              | <0.01  | <0.01  |
| CE                                     | r <sup>y</sup>                            | <0.01          | <0.01  | <0.01  |

Table 7-2. Rosenfeld Numerical Terminology for Rh Antigens

| Numeric | Symbol                       | Numeric | Symbol            | Numeric | Symbol |
|---------|------------------------------|---------|-------------------|---------|--------|
| Rh1     | D                            | Rh27    | cE                | Rh48    | JAL    |
| Rh2     | C                            | Rh28    | hr <sup>H</sup>   | Rh49    | STEM   |
| Rh3     | E                            | Rh29    | "total"           | Rh50    | FPTT   |
| Rh4     | c                            | Rh30    | Go <sup>a</sup>   | Rh51    | MAR    |
| Rh5     | e                            | Rh31    | hr <sup>B</sup>   | Rh52    | BARC   |
| Rh6     | ce or f                      | Rh32    | Rh32 <sup>+</sup> | Rh53    | JAHK   |
| Rh7     | Ce                           | Rh33    | Rh33 <sup>+</sup> | Rh54    | DAK    |
| Rh8     | C <sup>w</sup>               | Rh34    | Hr <sup>B</sup>   | Rh55    | LOCR   |
| Rh9     | C <sup>x</sup>               | Rh35    | Rh35 <sup>§</sup> | Rh56    | CENR   |
| Rh10    | V                            | Rh36    | Be <sup>a</sup>   | Rh57    | CEST   |
| Rh11    | E <sup>w</sup>               | Rh37    | Evans             |         |        |
| Rh12    | G                            | Rh39    | Rh39              |         |        |
| Rh17    | Hr <sub>0</sub> <sup>*</sup> | Rh40    | Tar               |         |        |
| Rh18    | Hr                           | Rh41    | Rh41              |         |        |
| Rh19    | hr <sup>s</sup>              | Rh42    | Rh42              |         |        |
| Rh20    | VS                           | Rh43    | Crawford          |         |        |
| Rh21    | C <sup>G</sup>               | Rh44    | Nou               |         |        |
| Rh22    | CE                           | Rh45    | Riv               |         |        |
| Rh23    | D <sup>w</sup>               | Rh46    | Sec               |         |        |
| Rh26    | c-like                       | Rh47    | Dav               |         |        |

Note: Rh13 through 16, 24, and 25 are obsolete.  
\*High-frequency antigen. The antibody is made by D—/D— and similar phenotypes.  
†Low-incidence antigen expressed by R<sup>N</sup> and DBT phenotypes.  
‡Original described on R<sub>0</sub>Har phenotype but also found on R<sup>N</sup>, D<sup>Via</sup>(C)—, R<sub>0</sub><sup>JoH</sup>, R<sub>1</sub><sup>Lisa</sup>.  
§Low-frequency antigen on D(C)(E) cell.

The major Rh antigens are D, C, c, E, and e, but the Rh blood group system is one of the most complex because of the number of additional antigens that have been reported (Table 7-2). These additional antigens include compound antigens in *cis* [eg, f (ce), Ce, CE], low-incidence antigens arising from partial-D hybrid proteins (eg, D<sup>w</sup>, Go<sup>a</sup>, Evans), and antigens arising from various point mutations in the RhCE protein (eg, C<sup>w</sup>, C<sup>x</sup>, VS). Table 7-2 also includes the numeric designations for Rh antigens that Rosenfeld introduced in 1962 in anticipation

of the computer era.<sup>7</sup> With a few exceptions (Rh17, Rh29), the numeric designations are not widely used in the clinical laboratory.

Genes and Their Expressed Proteins

Two genes designated *RHD* and *RHCE* encode the Rh proteins.<sup>8</sup> They are 97% identical, each has 10 exons, and they are the result of a gene duplication on chromosome 1p34-36.<sup>9</sup> Rh-positive individuals have both genes, while most Rh-negative individuals have only the *RHCE* gene (see below).

RhD and RhCE are 417-amino acid, nonglycosylated proteins. One protein carries the D antigen, and the other carries various combinations of the CE antigens (ce, cE, Ce, or CE). RhD differs from RhCE by 32 to 35 amino acids, depending on which form of RhCE is present (Fig 7-1).<sup>10-13</sup> This degree of difference explains why D is the most immunogenic of all the blood group proteins, because most other blood group antigen polymorphisms result from single amino acid changes in the respective protein. The Rh proteins migrate in sodium dodecyl sulphate polyacrylamide gels with an approximate *M<sub>r</sub>* of 30 to 32kD and hence are sometimes referred to as the Rh30 proteins. They are predicted to span the membrane 12 times and are covalently linked to fatty acids (palmitate) in the lipid bilayer (Fig 7-1).<sup>14</sup>

Molecular Basis for Antigen Expression  
D Antigen

“Rh positive” and “Rh negative” refer to the presence or absence of the D antigen, respectively. The Rh-negative phenotype occurs in 15% to 17% of Whites, but is not as common in other ethnic populations.<sup>4(p195)</sup> The absence of D in people of European ancestry was caused by a complete deletion of the *RHD* gene<sup>15</sup> and probably occurred on a D<sub>ce</sub> (R<sub>0</sub>) haplotype because the allele most often carried with the deletion is *ce*. In contrast, inactive or silent *RHD* rather than a complete gene deletion causes D-negative phenotypes in Asians or Africans. D-negative phenotypes in Asians occur with a frequency of <1%,<sup>4(p195)</sup> and most carry mutant *RHD* genes associated with *Ce*, indicating that they probably originated on a D<sub>Ce</sub> (R<sub>1</sub>) haplotype. Only 3% to 7% of South African Blacks are D-negative, but 66% have *RHD* genes that contain a 37-bp internal duplication, which results in a premature stop codon. The 37-bp insert *RHD*-pseudogene was also found in 24% of D-negative African-Americans. Additionally, 15% of D-negative phenotypes in Africans result from a hybrid *RHD-CE-D* gene that does not encode D epitopes. In total, only 18% of D-negative Africans and 54% of D-negative African-Americans completely lack *RHD*.<sup>16</sup> This is important when designing polymerase chain reaction (PCR)-based methods to predict the D status of the fetus and the possibility of HDFN. The population being tested and the different molecular events responsible for D-negative phenotypes must be considered. Even among D-negative Whites, rare cases of an *RHD* gene that is not expressed because of mutation or bp-insertions have been reported.<sup>17</sup>

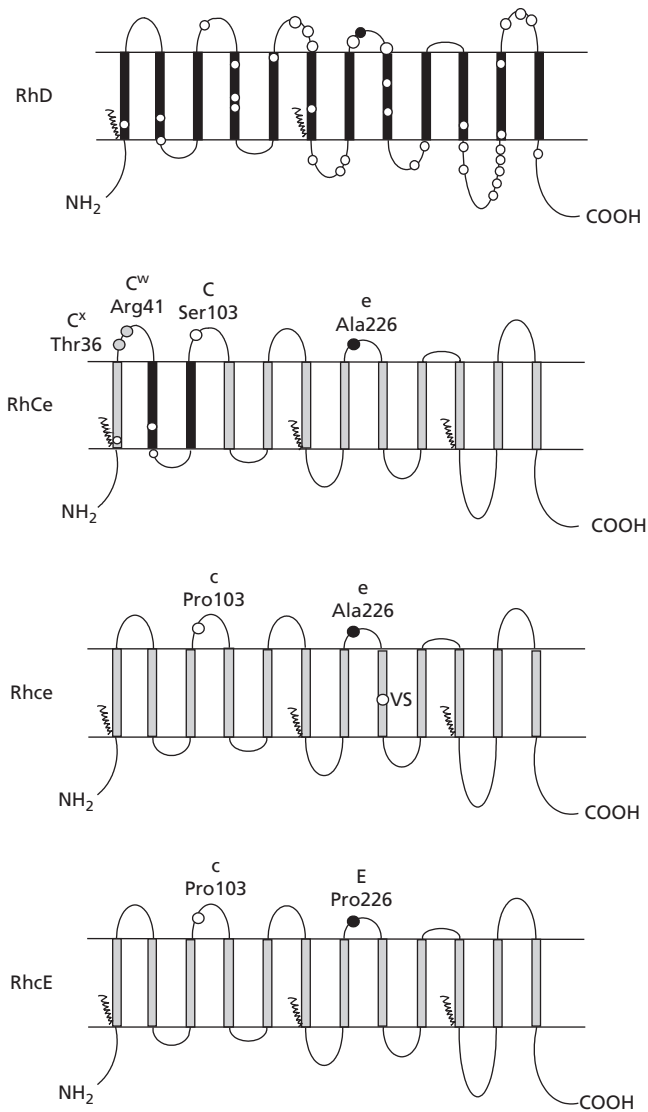


**Figure 7-1.** Predicted membrane topology of RhD and the major RhCE proteins. The amino and carboxy termini are cytoplasmic and the proteins are predicted to transverse the membrane 12 times. The location of the amino acid residues that differ between D and CE are represented by open circles, only nine of which are predicted to be extracellular. The C/c (Ser103Pro) polymorphism located on the second extracellular loop and the e/E (Ala226Pro) polymorphism on the fourth extracellular loop are shown. The shared region of RhD and RhCe responsible for expression of the G antigen is shown in black. Amino acid changes responsible for C<sup>x</sup> and C<sup>w</sup> are located on the first extracellular loop and the VS antigen, common in Blacks, is located in the eighth transmembrane domain of Rhce. The zigzag lines represent covalent linkage to fatty acid in the lipid bilayer.

### Weak D

An estimated 0.2% to 1% of Whites (and a greater number of Blacks) have reduced expression of the D antigen,<sup>4(p407)</sup> which is characterized serologically as failure of such red cells to agglutinate directly with anti-D typing reagents, requiring the use of an indirect antiglobulin test for detection. The molecular basis

of weak-D expression is heterogeneous and is associated with the presence of point mutations in *RHD*. The mutations cause amino acid changes predicted to be intracellular or in the transmembrane regions of RhD rather than on the outer surface of the red cell (Fig 7-2).<sup>18</sup> This suggests that these mutations affect the efficiency of insertion and, therefore, the quantity of protein in the membrane, and may not affect the expression of D epitopes. This explains why most individuals with a weak-D phenotype can safely receive D-positive blood and do not make anti-D. Exceptions occur, however, and include individuals who make D antibodies that cross-react with their own red cells and appear to be autoantibodies, others that make alloanti-D, or both. There are over 50 different mutations known to cause weak-D expression (Type 1 through Type 54). The long history of transfusing patients who have weak-D red cells with D-positive blood suggests that weak-D Types 1, 2, and 3 (which represent the majority of Whites with weak D) are unlikely to make anti-D. Nevertheless, some have made anti-D, suggesting they have altered D epitopes. Predicting which mutations alter D epitopes is an active area of investigation.<sup>19,20</sup>

It is important that donor center typing procedures detect and label weak-D Red Blood Cell (RBC) units as D-positive, because they can stimulate D-negative recipients. However, weak-D typing is not required for transfusion recipients, who would then receive D-negative units without untoward effects.

A very weak form of D expression (D<sub>el</sub>), which cannot be detected by routine serology methods but can be demonstrated by adsorbing and eluting anti-D, is not uncommon in Asians (10-30% of apparent D-negative). Red cells with very low levels of D are primarily of concern for donor testing, because they have stimulated anti-D in D-negative recipients.<sup>21</sup>

### Partial D

The D antigen has long been described as a “mosaic” because of the observation that some Rh-positive individuals produce anti-D when exposed to D antigen. It was hypothesized that the red cells of these individuals lack some part of D and that they can produce antibodies to the missing portion. Molecular analysis has shown that this is correct, but what was not predicted is that the missing portions of *RHD* are replaced by corresponding portions of *RHCE* in the great majority of cases (Fig 7-3).<sup>17,20</sup> The novel sequences of amino acids and the conformational changes that result from segments of RhD joined to segments of RhCE can generate new antigens (eg, BARC, D<sup>w</sup>, FPTT, DAK, Go<sup>a</sup>, Evans, Rh32) (Fig 7-3 and Table 7-2). The replacements are the result of gene conversion, the hallmark being that the donor gene is unchanged. Replacements involve single or multiple exons, while others involve short stretches of amino acids (Fig 7-3). Some also result from only single amino acid changes (eg, DMH, DFW, D<sup>II</sup>) (Fig 7-2). In contrast to the single amino acid changes that cause weak D (above), which are predicted to be cytoplasmic or transmembrane in location, those that cause partial-D phenotypes are predicted to be located on the extracellular loops of the protein (Fig 7-2). The extracellular location



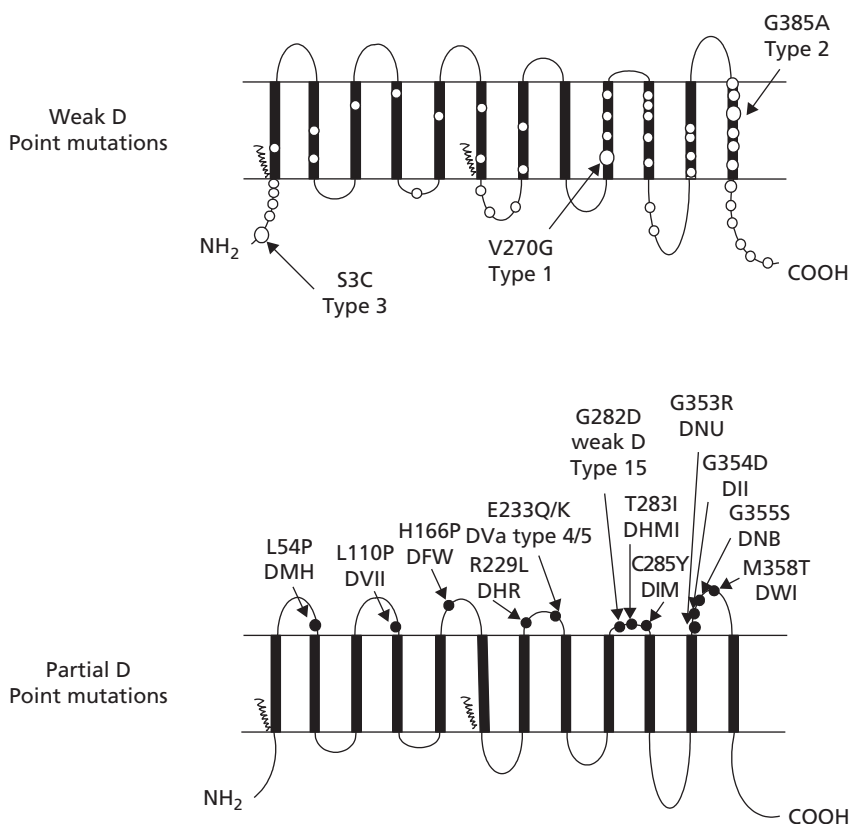


**Figure 7-2.** Predicted location of point mutations that cause weak D and partial-D phenotypes. Weak D phenotypes carry mutations in *RHD* that primarily cause amino acid changes predicted to be intracellular or in the transmembrane regions of D (upper panel). The specific amino acid mutation and the location in the protein are indicated. The partial D phenotypes that carry mutations in *RHD* are predicted to be located on extracellular loops of RhD (lower panel). The specific amino acid mutation and location in the protein, as well as the common name for each are indicated.

of the changes explains why these individuals recognize conventional D as foreign. From a clinical standpoint, individuals with partial-D antigens ideally should receive D-negative blood, especially females of childbearing potential, but in practice most will type as D-positive (especially if a weak-D test is performed) and will be recognized only after they have made anti-D following a transfusion with D-positive cells.

### Elevated D

Several phenotypes, including D<sup>-</sup>, D<sup>c</sup>-, and DC<sup>w</sup>-, have enhanced expression of D antigen and no, weak, or variant CE antigens, respectively.<sup>4(p407)</sup> These phenotypes are analogous to the partial-D rearrangements described above, only they involve the opposite situation—that is, replacement of portions of *RHCE* by *RHD* (Fig 7-3). The additional *RHD* sequences in *RHCE* along with a normal *RHD* explain the enhanced D and account for the reduced or missing CE antigens. Although these represent altered *RHCE* genes (see below), they are included here because of their elevated D phenotype. Individuals with such altered CE phenotypes can make anti-Rh17 when immunized.

### C/c and E/e Antigens

The four major forms of the *RHCE* gene encode four different proteins: RhCe, -ce, -cE and -CE (Fig 7-1).<sup>12</sup> C and c differ by four amino acids: Cys16Trp encoded by exon 1, and Ile60Leu, Ser68Asn, and Ser103Pro encoded by exon 2 (Fig 7-1, open circles

on RhCe). Of those four amino acids, only the residue at 103 is predicted to be extracellular and is located on the second loop. All the amino acids encoded by exon 2 of *RHCE* are identical to those encoded by exon 2 of *RHD* (Fig 7-1, black on RhCe). This suggests that *RHCE* arose from the transfer of exon 2 from an *RHD* into an *RHCE* gene, respectively. The sharing of exon 2 encoded amino acids by *RHD*, *RHCE*, and *RHCE* accounts for the expression of the G antigen on red cells that are D or C positive. E and e differ by one amino acid, Pro226Ala, predicted to reside on the fourth extracellular loop of the protein (Fig 7-1, solid circle). The E antigen arose from a single point mutation that occurred in exon 5 of a *RHCE* gene, giving rise to *RHCE*.

### Altered CE

C<sup>w</sup> and C<sup>x</sup> are low-incidence antigens that result from single amino acid changes (Gln41Arg and Ala36Thr, respectively) predicted to be located on the first extracellular loop of RhCE (Fig 7-1, gray circles).<sup>22</sup> These antigens are more common in Finns (4%), and are most often present on RhCe. C<sup>w</sup> is also associated with the deletion phenotype DC<sup>w</sup>- (Fig 7-3).

V and VS antigens, which are expressed on red cells of more than 30% of Blacks, result from a Leu245Val substitution located in the predicted eighth transmembrane segment of Rhce (Fig 7-1).<sup>23</sup> The V–VS+ phenotype results from a Gly336Cys change on the 245Val background.<sup>24</sup> V+ and VS+ are associated with weak and altered expression of e, indicating that Leu245Val probably



**Figure 7-3.** Gene conversion events between *RHCE* and *RHD* produce chimeric Rh proteins. Gray (*RHCE*) and black boxes (*RHD*) represent the 10 exons that encode Rh polypeptides. Gene conversion events involve amino acids (vertical bars) or whole exons (filled boxes). Replacement of portions of *RHD* by *RHCE* (upper set of panels) causes many of the partial-D phenotypes, some of which are shown here. Replacement of portions of *RHCE* by *RHD* cause elevated D phenotypes with concurrent loss of expression of CE antigens (middle panel) or altered CE expression (lower set of panels).

causes a local conformation change on the fourth extracellular loop where the e-specific amino acid resides.

Most individuals have e-positive red cells, but the e antigen is considered to be second in complexity to D because variant expression has frequently been observed.<sup>25</sup> The e antigen is altered on red cells with the following alleles—DHar, ceSL, R<sup>N</sup>, ceAR, ceEK (hr<sup>S</sup>-), ce<sup>S</sup> (hr<sup>B</sup>-), and ceMO (hr<sup>S</sup>-, hr<sup>B</sup>-) (Fig 7-3). Extensive discussion is beyond the scope of this chapter, but the majority are found in African ethnic groups, and consequently, are prevalent in patients with sickle cell disease (SCD).<sup>20,22,26</sup> These patients not infrequently produce anti-e following transfusion, despite having an e-positive red cell phenotype. Altered expression of e also results from loss of the codon for Arg229,<sup>17</sup> and is also associated with the presence of a 16Cys residue in Rhce, which occurs frequently on the R<sub>0</sub> haplotype common in African-Americans.<sup>27</sup>

E variants are not common and include EI, EII, and EIII, which result from a point mutation (EI) or gene conversion replacement

of RhcE amino acids with RhD residues (EII and EIII) with concurrent loss of some E epitope expression. Category EIV red cells, which have an amino acid substitution in an intracellular domain, do not lack E epitopes but have reduced E expression.<sup>28</sup>

Variants of c are infrequent. The very rare RH:-26 results from a Gly96Ser transmembrane amino acid change that abolishes Rh26 and weakens c expression.<sup>29</sup> The lack of c antigen variants in humans compared to the other Rh antigens, and the preservation of expression of c on the red cells of nonhuman primates suggest that the two proline residues involved form a stable structure that is resistant to perturbations and changes in Rhce.<sup>30</sup>

In summary, point mutations and genetic exchange, mainly involving gene conversion events between *RHD* and *RHCE*, are primarily responsible for the large number of Rh antigens. Additional complexity results because many of the Rh epitopes are highly conformational and single amino acid changes in one part of the protein, including changes within the transmembrane

regions, can affect the expression of cell-surface-exposed antigen epitopes.

### RH Genotyping

DNA-based molecular testing methods were introduced to the blood bank and transfusion medicine community approximately a decade ago, and much progress has been made in designing reproducible assays and validating gene targets. Assays for blood group antigens encoded by single nucleotide polymorphisms (SNPs) are highly reproducible and correlate with red cell phenotype. Genotyping for the two most important blood group systems, ABO and Rh, are more challenging because of the many different mutations that cause weak expression of A and B, and inactive O, and because of the numerous variant and hybrid *RH* alleles. Multiple regions of the genes must be sampled and hybrid genes are problematic for analysis. Genotyping for blood group antigens is performed principally in a reference laboratory setting, although testing platforms that used bead or microarray technology hold promise for implementation of high-throughput *RH* genotyping for donors and patients.<sup>31</sup>

### RHD Zygosity Testing

Serologic testing for red cell expression of D, C/c, and E/e can only predict the likelihood that a sample is homozygous (D/D) or heterozygous (D/–) for *RHD*. Genotyping enables zygosity to be determined by assaying for the presence of a recessive D-negative allele. In prenatal practice, paternal *RHD* zygosity testing is important to predict the fetal D status when the mother has anti-D. Several different genetic events cause a D-negative phenotype, and multiple assays must be performed to accurately determine zygosity. These include detection of the region generated by deletion of *RHD*, and the 37-bp insert *RHD* pseudogene or the D-negative *RHD-CE-D* hybrid gene common in African Black ethnic groups. If the father is *RHD* homozygous, the fetus will be D-positive, and monitoring of the pregnancy will be required. If the father is heterozygous, the D type of the fetus should be determined to prevent invasive and unnecessary testing.

### Fetal Typing

Genotyping is important in the prenatal setting to determine whether the fetus has inherited the paternal antigen to which the mother has a clinically significant antibody. Fetal DNA can be isolated from cells obtained by amniocentesis or chorionic villus sampling. Alternatively, the discovery that cell-free, fetal-derived DNA is present in maternal plasma or serum by approximately 5 weeks' gestation allows maternal plasma to be used as a source of fetal DNA.<sup>32</sup> Fetal DNA in maternal plasma is derived from apoptotic syncytiotrophoblasts, increases in concentration with gestational age, and is rapidly cleared following delivery.<sup>33</sup> The small quantity of cell-free fetal DNA present relative to maternal DNA poses a challenge, and positive controls for isolation of sufficient fetal DNA are critical to validate negative results. When combined with sensitive real-time PCR methodology, isolation of fetal DNA from maternal plasma has been successfully applied

to determine fetal D status.<sup>34</sup> This has been more successful for D typing because the D-negative phenotype in the majority of samples is caused by the lack of *RHD*. Testing for the presence or absence of a gene is less demanding than testing for a single gene polymorphism or SNP. Theoretically, testing the maternal plasma for the presence of a fetal *RHD* could be used to eliminate the unnecessary administration of antepartum Rh Immune Globulin (RhIG) to the approximately 40% of D-negative women who are carrying a D-negative fetus. RhIG is not entirely risk free, and this approach would be cost-effective for some health-care systems.<sup>34</sup>

### Distinction between Weak D and Partial D

As indicated above, altered expression of D antigen is not uncommon. Weak D phenotypes have single amino acid changes that primarily affect the quantity of RhD in the membrane. Partial D phenotypes have amino acid changes that alter D epitopes, or often are hybrid proteins with portions of RhD joined to portions of RhCE. The distinction between weak D and partial D phenotypes is of clinical importance because the latter make anti-D. Routine serologic typing reagents cannot distinguish between these red cells; however, genotyping strategies that sample multiple regions of *RHD* can discriminate between weak D and partial D.

### Detecting Patients at Risk for Production of Antibodies to High-Incidence Antigens

Alloimmunization is a serious complication of chronic transfusion, particularly in patients with SCD requiring long-term transfusion support. Many transfusion programs attempt to prevent or reduce the risk and incidence of alloantibody production in SCD patients by transfusing RBC units that are antigen-matched for D, C, E, and Kell. Although this approach reduces the incidence of alloimmunization, variant *RHD* and *RHce* genes are common in African Blacks and individuals of mixed ethnic backgrounds. The prevalence of *RH* alleles that encode altered D, C, and e antigens in this patient group explains why some SCD patients become immunized to Rh, despite Rh antigen-matching for D, C, and E.<sup>20,35</sup> These antibodies often have complex, high-incidence Rh specificities, and it can be difficult or impossible to find compatible units. Genotyping can identify those patients who are homozygous for variant *RH* alleles and at risk for production of alloantibodies to high-incidence Rh antigens and can identify compatible donors for transfusion.

### Rh<sub>null</sub> Phenotype

Rh<sub>null</sub> individuals lack expression of all Rh antigens. They suffer from a compensated hemolytic anemia, have variable degrees of spherocytosis, stomatocytosis, and increased red cell osmotic fragility.<sup>36</sup> The phenotype is rare and occurs on two different genetic backgrounds: the “regulator” type, caused by a gene at an unlinked locus, and the “amorph” type, which maps to the *RH* locus.<sup>5</sup> It is now clear that in the more common, regulator type of Rh<sub>null</sub>, the suppression of Rh is caused by a lack of, or a

mutant, Rh-associated glycoprotein (RhAG), also called Rh50 protein.<sup>37</sup> RhAG is a 409-amino acid glycosylated protein that coprecipitates with RhD and RhCE.<sup>38</sup> It shares 37% amino acid identity with the RhD/RhCE proteins and has the same predicted membrane topology. RhAG is not polymorphic and does not carry Rh antigens; however, it is important for targeting the Rh proteins to the membrane during erythroid development. RhAG has one N-glycan chain that carries ABO and Ii specificities.<sup>39</sup> It is encoded by a single gene, *RHAG*, located at chromosome 6p11-21.1.<sup>38</sup>

The amorph type of Rh<sub>null</sub> results from mutations in *RHCE* on a D-negative background.<sup>40</sup> Amorph-type red cells express no Rh protein and have reduced amounts (~20%) of RhAG.

### Rh-Membrane Complex

Additional complication in Rh protein structures arises because they exist in the red cell membrane as complexes with several other proteins. Rh and RhAG are associated in the membrane as a core complex.<sup>41</sup> The fact that several other proteins interact with the Rh-core complex is based on observations of Rh<sub>null</sub> cells. These red cells have reduced expression of CD47, an integrin-associated protein (IAP) that has wide tissue distribution, binds  $\beta_3$  integrins, and is required for integrin-regulated  $\text{Ca}^{2+}$  entry into endothelial cells. Its function on the red cells is unknown; however, a role in red cell senescence has been suggested.<sup>42</sup> Rh<sub>null</sub> cells also have reduced glyophorin B (GPB), a sialoglycoprotein that carries S or s and U antigens. GPB appears to aid RhAG trafficking to the membrane, because the RhAG protein in GPB-deficient cells has increased glycosylation, reflecting longer dwell time in the endoplasmic reticulum. Rh<sub>null</sub> cells also lack LW, a glycoprotein of unknown function that belongs to the family of intercellular adhesion molecules (ICAM-4, see below). Band 3 (the anion exchanger) enhances the expression of the Rh antigens in transfected cells, suggesting that band 3 may also be associated with the Rh-core complex.<sup>43</sup> Recent studies reveal that the Rh-core complex is linked to the membrane skeleton through interactions between CD47 and protein 4.2<sup>44</sup> and through a novel Rh/RhAG-ankyrin cytoskeleton connection.<sup>45</sup> Red cell membrane protein-cytoskeleton and protein-protein interactions are an active area of investigation.

### Rh Function

#### Rh Glycoproteins (RhAG, RhBG, RhCG)

The Rh blood group proteins are well known because of their importance in blood transfusion. However, the mammalian family of Rh proteins has been expanded with the discovery of RhAG in erythrocytes, and the related proteins, RhBG and RhCG, in other tissues.

Protein sequences with similarities to the mammalian Rh proteins were first found in *Caenorhabditis elegans*, and these homologues, in turn, showed similarity to the ammonia transporters from bacteria, yeast (MEP), and plants (AMT).<sup>46</sup> The relationship of the Rh glycoproteins to the AMT/MEP ammonia transporters from these other organisms has been substantiated

by functional transport data<sup>47-49</sup> and structural modeling.<sup>50,51</sup> The Rh proteins reveal the power of comparative genomics and proteomics, in which sequence analysis and homology modeling can give important insight into mammalian protein function.

The nonerythroid Rh glycoproteins, RhBG and RhCG, are present in the kidney, liver, brain, and skin where ammonia production and elimination occur. In the kidney collecting segment and collecting duct, RhBG and RhCG are found on the basolateral and apical membranes, respectively, of the intercalated cells where they mediate transepithelial movement of ammonia from the interstitium to the lumen.<sup>52</sup> In the liver, RhBG is found on the basolateral membrane of perivenous hepatocytes, where it may function in ammonia uptake. RhCG is also present in bile duct epithelial cells, where it is positioned to contribute to ammonia secretion into the bile fluid.<sup>53</sup>

The mechanism of ammonia transport by Rh glycoproteins is an active area of investigation. Expression of Rh glycoproteins in heterologous systems indicates that mammalian transport is an electroneutral process that is driven by the  $\text{NH}_4^+$  concentration and the transmembrane  $\text{H}^+$  gradient.<sup>54,55</sup> Functional studies of the kidney, liver, and brain Rh homologues, along with the erythrocyte RhAG/Rh proteins, promise to lead to development of a unifying hypothesis of ammonia transport in mammals by the Rh family of proteins.

### RhCE and RhD

The function of the more recently evolved erythrocyte blood group proteins, RhCE and RhD, has not yet been determined. When expressed in heterologous systems, they do not directly transport ammonia.<sup>56</sup> RhCE and RhD lack the highly conserved histidine residues located in the membrane pore that are critical for ammonia transport. RhCE and RhD may be evolving a new function in the red cell membrane as phylogenetic analysis indicates the RhCE/D proteins are rapidly evolving, suggesting their function may be changing.

### Immune Response to Rh

#### Medical Aspects

Human red cells can express more than 400 different blood group antigens. Typing patient and donor cells for every known antigen with the intention of providing perfectly matched blood would be a practical and fiscal impossibility. Fortunately, such extensive testing is not required for a number of reasons, the most important of which is that exposure to the majority of foreign red cell antigens through transfusion does not lead to the production of clinically significant alloantibodies. D is one notable exception. As many as 80% of D-negative patients exposed to D-positive red cells may develop high-titer, high-affinity, D IgG antibodies that may persist for the rest of their lives even if they are never exposed to the antigen again. The antibodies can cause HTRs and can cross the placenta causing HDFN when present in a D-negative female carrying a D-positive fetus. Therefore, because of its extraordinary immunogenicity and clinical significance, the D antigen is the only blood group antigen for which

it is routine to prophylactically match blood before transfusion so as to avoid immunization. Although antibodies to C, c, E, and e can cause HTRs and HDFN, they are much less immunogenic than D (~1% rate of sensitization) and the use of RBCs lacking one or more of those antigens are indicated for patients only after sensitization has occurred (management of sickle cell patients may differ as described in preceding section).

In practice, D-positive patients can be transfused with either D-positive or D-negative RBCs—the absence of D will cause no harm—but it is deemed prudent to reserve the rarer units of D-negative blood (~15% of donor units) for D-negative individuals who must receive them. In cases of trauma and/or massive transfusion in which the patient's D status is unknown, efforts are made to provide D-negative blood, especially for females of childbearing potential, until the appropriate testing can be completed. When D-negative blood is in short or critical supply, it may be necessary to transfuse D-negative patients with D-positive units. In such scenarios, D-negative units are reserved for females of childbearing potential and for patients whose serum contains anti-D from a previous sensitization.

Unlike ABO blood group antigens, which are expressed by all transfused blood cells including platelets, the D antigen is present only on red cells. Theoretically, the selection of platelet units for transfusion should be independent of the D status of the donor. However, a transfusion of pooled platelet concentrates may introduce as much as 5 mL of donor red cells, which may be sufficient to alloimmunize a D-negative patient. Therefore, the standard of care is to avoid transfusing D-negative patients, particularly females of childbearing potential, with platelet units derived from D-positive donors. If such units are unavailable and platelet transfusion must be undertaken, the administration of RhIG can be considered. A standard 300- $\mu$ g dose of RhIG, which may inhibit the immunizing potential of up to 15 mL of D-positive red cells, would neutralize the effects of D-positive red cells from several mismatched platelet transfusions. With respect to the transfusion of plasma products, the D status of the donor is not an issue because plasma products do not contain cellular or soluble material capable of inducing anti-D immune responses.

### Serologic Aspects

The immune response to Rh, like that to other peptide antigens, is typically thymus-dependent, requiring T-cell help. Upon exposure to a foreign Rh antigen, an IgM response may develop, but this is quickly followed by the production of IgG antibodies. Consequently, nearly all examples of Rh antibodies are IgG molecules (mostly IgG1 and IgG3), which bind optimally to red cells at 37°C and require the addition of an antiglobulin reagent to produce hemagglutination. Although IgG1 and IgG3 subclasses classically initiate complement activation, the vast majority of anti-Rh-containing sera do not do so. The usual explanation for this cites the relatively low copy number of Rh antigens per red cell, which results in Rh molecules situated too far apart on the cell surface to permit the simultaneous binding of C1q by multiple Rh IgG antibodies. Therefore, hemolysis from the transfusion

of Rh-incompatible RBCs is generally extravascular because of the phagocytosis of IgG-coated erythrocytes by cells of the reticuloendothelial system.

After anti-D, the Rh antibodies most commonly found in the sera of alloimmunized individuals are anti-E > anti-c > anti-e > anti-C. In approximately 50% of cases of warm-type autoimmune hemolytic anemia (WAIHA), autoantibodies are believed to be directed to Rh antigens by virtue of their “panreactivity” with all red cell phenotypes except Rh<sub>null</sub> cells. However, direct binding of autoantibodies to putative epitopes common to D and C/E polypeptides or to other components of the Rh-membrane complex (RhAG, CD47, etc) has yet to be demonstrated in WAIHA. The difficulties in approaching this problem are largely technical in nature and relate to both the inability to produce workable quantities of pure patient autoantibody *in vitro* (ie, clone the autoantibody-producing B lymphocytes) and the inability to purify Rh proteins in a way that retains their native conformationally dependent epitopes.

### Molecular Aspects

The characterization of Rh antibodies on a molecular level, particularly that of anti-D, has been the focus of much study not only because of their clinical significance, but also because of the need to develop suitable *in-vitro* methods for their production.<sup>57</sup> Ironically, because of better transfusion practice and the use of RhIG, alloimmunization of antigen-negative individuals is significantly less common (as are sera donors willing to be purposely hyperimmunized), so that supplies of Rh antibodies for use as typing reagents and for the preparation of RhIG are dwindling. To better understand the molecular make-up of Rh antibodies, investigations have focused on analyzing their variable regions in order to determine whether there are commonly shared genetic and/or structural features among Rh antibodies made by different individuals.

Early work using rabbit antisera specific for different human heavy-chain variable region gene products suggested a restriction in the use of certain heavy-chain gene families by the anti-D contained in polyclonal sera from several dozen anti-D donors.<sup>58</sup> Subsequent studies with rodent idiotypic antibodies demonstrated cross-reactive idiotypes among polyclonal anti-D preparations<sup>59,60</sup> and among different examples of human monoclonal anti-C, -c, -D, -E, -e, and -G produced by transformed B cells.<sup>61</sup> A more direct approach using nucleotide sequencing to examine immunoglobulin gene diversity examined a cohort of four IgM and 10 IgG monoclonal anti-D variable regions.<sup>62</sup> A restricted use of the human heavy-chain variable region genes *V<sub>H</sub>3-33* and *V<sub>H</sub>4-34* was found with a shift in repertoire usage toward *V<sub>H</sub>3-33* for anti-D that had isotype switched to the more clinically relevant IgG. The restriction of anti-D heavy chains to the use of these and other highly related *V<sub>H</sub>* genes has been confirmed and extended through the analysis of many additional examples of anti-D produced through both tissue culture and recombinant means.<sup>63-68</sup> The use of molecular approaches such as site-directed mutagenesis,<sup>69</sup> complementarity-determining region (CDR) sequence

randomization,<sup>70</sup> and heavy-chain/light-chain “shuffling”<sup>71</sup> has demonstrated the genetic relatedness among anti-D molecules directed against different D epitopes as well as among antibodies with D and E specificity. These studies and others<sup>72,73</sup> have supported the hypothesis that a restricted “Rh footprint” for D alloantibodies and a process termed “epitope migration”<sup>63</sup> play a role in the molding of the anti-Rh immune repertoire.<sup>74</sup>

Although the precise significance of immunoglobulin germline gene restriction by Rh antibodies is not fully understood, it may have practical significance for the preparation of anti-D for both therapeutic and diagnostic use. For example, the  $V_H$  genes used to encode anti-D are among the most cationic of the human germline  $V_H$  genes<sup>75</sup> and may account for the relatively high pI of polyclonal anti-D-containing antisera originally noted over 40 years ago.<sup>76</sup> Although the cationic nature of the antibodies may be important for binding to D, it has also been suggested that a constitutive net-positive charge may be necessary to permeate the highly negative red cell zeta potential, thus permitting antibody to contact antigen.<sup>77</sup> Second, while IgM D monoclonal antibodies are well-suited for antigen typing because they may serve as direct agglutinins, the fact that they are most often encoded by  $V_H4-34$  (the germline gene to which cold agglutinins are also restricted),<sup>78,79</sup> may explain why many IgM monoclonal anti-D typing reagents falsely agglutinate D-negative cells when used at cooler-than-recommended temperatures. This phenomenon may also explain the body of literature claiming that the D antigen was present on numerous nonerythroid cells. Using IgM anti-D, these investigators may have been detecting antigens of the I/i blood group system.

## LW Blood Group System

### History and Nomenclature

The LW antigens are the “true” Rhesus antigens shared by humans and the rhesus monkey. As discussed earlier, the confusion occurred because LW antigens are more abundant on D-positive than on D-negative red cells. When the situation was clarified, the term “Rh” remained associated with the human antigen, so the real Rhesus antigen was renamed LW in honor of Landsteiner and Wiener.<sup>5</sup> The confusion can be understood today in the transfusion service when a weak example of anti-LW<sup>a</sup> often appears initially to be anti-D. The LW system has undergone additional terminology revisions. The historical terminology of LW<sub>1</sub>, LW<sub>2</sub>, LW<sub>3</sub>, LW<sub>4</sub>, and LW<sub>0</sub> to describe phenotypes was based on both the LW and the D status of the red cells but is now obsolete.<sup>5</sup> The phenotypes are LW(a+b−), LW(a−b+), LW(a+b+), and the rare LW(a−b−); the antigens are designated LW<sup>a</sup>, LW<sup>b</sup>, and LW<sup>ab</sup>.

### Genes and Their Expressed Proteins

LW is encoded by a single gene located on chromosome 19. The 42-kD LW glycoprotein is a member of the family of ICAMs and has been renamed ICAM-4 (CD242). LW passes through

the red cell membrane once, and the N-terminal extracellular region is organized into two immunoglobulin superfamily (IgSF) domains.<sup>80</sup>

### Molecular Basis for Antigen Expression

LW<sup>a</sup> is the common antigen, whereas LW<sup>b</sup> has an incidence of less than 1% in most Europeans.<sup>4(p407)</sup> The LW<sup>a</sup>/LW<sup>b</sup> polymorphism is caused by a single amino acid substitution, Gln70Arg, on the LW glycoprotein.<sup>81</sup> An increased frequency of the uncommon LW<sup>b</sup> antigen in Latvians and Lithuanians (6%), Estonians (4%), Finns (3%), and Poles (2%) suggests that the LW<sup>b</sup> mutation originated in the people of the Baltic region. The LW<sup>ab</sup> antigen was originally defined by an alloantibody made by the only known (genetically verified) LW(a−b−) person, who lacks expression of all LW antigens.<sup>5</sup> The LW gene in this rare LW(a−b−) individual has a 10-bp deletion and a premature stop codon in the first exon.<sup>82</sup> Rh<sub>null</sub> red cells also lack LW antigens but do not have defective LW genes. Rh proteins appear to be required for LW to traffic to the membrane, and association with the D antigen is preferred.

LW antigens require divalent cations (eg, Mg<sup>2+</sup>) for expression and have intramolecular disulfide bonds that are sensitive to dithiothreitol (DTT) treatment.<sup>5</sup> This is helpful to differentiate anti-LW from anti-D, because the D antigen is resistant to DTT. Also helpful in identifying anti-LW is the fact that LW antigens are expressed equally well on D-positive and D-negative cord blood red cells.<sup>4(p407)</sup>

Transient loss of LW antigens has been described in pregnancy and patients with diseases, particularly Hodgkin disease, lymphoma, leukemia, sarcoma, and other forms of malignancy. Transient loss of LW antigens is associated with the production of autoanti-LW that can appear to be alloantibody.<sup>4(p407)</sup>

### LW Function

LW glycoprotein, ICAM-4, is a ligand with broad specificity for a number of  $\beta_1$ ,  $\beta_2$ ,  $\beta_3$ , and  $\beta_5$  integrins including  $\alpha_L\beta_2$  (LFA-1),  $\alpha_M\beta_2$  (Mac-1),  $\alpha_4\beta_1$  (VLA-4),  $\alpha_V\beta_1$ ,  $\alpha_V\beta_5$ , as well as platelet integrin  $\alpha_{2b}\beta_3$ .<sup>80,83-87</sup> In addition, LW binds to the I domains of CD11a/CD18 and CD11b/CD18 on leukocytes.<sup>84,88</sup> The function of LW glycoprotein on mature red cells is currently an area of active investigation. Evidence is emerging for both a normal homeostatic role in erythroblastic island formation through LW interaction with macrophage integrins<sup>89,90</sup> as well as pathophysiologic roles in the development of vaso-occlusion in SCD mediated by endothelial cell integrins<sup>91,92</sup> and of thrombosis mediated by activated integrins on platelets.<sup>87</sup>

## Summary

The molecular basis for the Rh antigens has now been elucidated. A gene deletion or silent *RHD* gene explains the absence of the D antigen in many Rh-negative individuals. The large number of amino acid differences between the RhD and RhCE proteins

explains why exposure in an individual lacking D often results in a vigorous immune response characterized by a very heterogeneous population of antibodies. The proximity of *RHD* and *RHCE*, duplicated genes on the same chromosome, has resulted in numerous exchanges by gene conversion between them. This has generated new polymorphisms and explains the many antigens observed in this blood group system. Molecular analysis has also revealed that Rh antigen expression is affected not only by changes in extracellular amino acids, but also by intracellular changes, highlighting the conformational nature of these blood group antigens and complicating attempts to map the epitopes to specific amino acid residues.

*RH* genotyping can be used to determine paternal *RHD* zygosity to predict HDFN and the fetus can be typed from amniocytes or from the maternal plasma. *RH* genotyping can also identify patients facing long-term transfusion therapy who are homozygous for variant *RH* alleles and at risk for production of alloantibodies to high-incidence Rh antigens. When partnered with *RH* genotyping of donors, this approach promises to have a positive impact on transfusion therapy outcomes by reducing alloimmunization, especially in patients with SCD.

Rh antibodies show restriction in their use of particular variable region immunoglobulin germline genes. Antibodies to serologically distinct epitopes may be genetically related, and epitope migration may be an important process that helps shape the composition of the anti-Rh immune repertoire. Questions remain concerning the function of the Rh proteins. The discovery that Rh protein homologues also exist in the liver and kidney indicates that the Rh blood group antigens belong to a larger, conserved family of proteins and will contribute to the elucidation of their function. Similarly, the function of LW on the mature red cell is not entirely clear but its ability to interact as an adhesion molecule with a broad range of integrin-binding specificity suggests an important role in both normal red cell development as well as disease-associated processes such as vaso-occlusion and unwanted thrombosis.

## Disclaimer

The authors have disclosed no conflicts of interest.

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# 8

## Other Protein Blood Groups

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Of the 29 currently known blood group systems, only seven have antigens formed by carbohydrates. Antigens of the remaining 22 blood group systems are carried by proteins. Two of these 22 systems—Rh (ISBT 004) and the closely associated LW (ISBT 016)—were discussed in detail in the previous chapter. The remaining blood group systems are summarized in Table 8-1.

Many of the proteins carrying blood group antigens are functionally important; however, antibodies in only a few blood group systems represent a problem for the transfusion service. This chapter addresses only those antigens that elicit formation of clinically significant antibodies and briefly comments on the other blood group systems. Further details may be found in review articles<sup>2,3</sup> and comprehensive textbooks.<sup>4-6</sup>

### Kell and Kx Blood Group Systems (ISBT 006 and 019)

#### Structure, Function, and Interaction of the Kell and XK Proteins

Antigens of the Kell blood group system are carried by a 93-kD red cell membrane glycoprotein.<sup>7</sup> The glycoprotein contains a short cytoplasmic N-terminal portion, a single membrane-spanning  $\alpha$ -helical segment, and a large, 665 amino acid extracellular C-terminal portion held in a globular conformation by multiple disulfide bonds (Fig 8-1). Kell antigens are inactivated by reducing agents such as dithiothreitol, suggesting that disulfide bonds are important in maintaining its antigenic conformation.<sup>8</sup> Proteolytic cleavage with chymotrypsin or trypsin also destroys Kell antigens.

The Kell protein is a member of the neprilysin (M13) family of zinc metalloproteases. This family consists of Kell, neutral endopeptidase 24.11, two different endothelin-converting

enzymes, the product of the *PEX* gene, and XCE.<sup>9,10</sup> Members of the M13 subfamily of membrane zinc endopeptidases have widely different roles, including processing of opioid peptides, Met- and Leu-enkephalin, oxytocin, bradykinin, angiotensin, endothelins, and parathyroid hormone. Kell protein has been shown to preferentially activate endothelin-3<sup>11</sup>; however, the in-vivo physiologic role of Kell protein is probably complex, because K<sub>0</sub> (null) persons are healthy.

The Kell protein interacts in the membrane with the 37-kD protein XK, which plays an important role in the expression of Kell system antigens. In contrast to the Kell protein, XK spans the membrane 10 times and both of its N- and C-termini are located intracellularly (Fig 8-1). The function of XK is not known. Structurally, XK resembles the glutamate transporters but it has very little amino acid sequence homology with this group of transport proteins. The Kell and XK proteins are covalently associated in the membrane by a disulfide link between cysteine 72 of Kell and cysteine 347 of XK<sup>12-14</sup> (Fig 8-1). The gene encoding the Kx antigen is located on the short arm of the X chromosome near the loci for X-linked chronic granulomatous disease (CGD) and Duchenne muscular dystrophy (DMD).

#### Kell in Transfusion Medicine

The Kell blood group system is the second most important protein blood group system in transfusion medicine after Rh, because the antibodies can cause hemolytic transfusion reactions (HTRs) and hemolytic disease of the fetus and newborn (HDFN). The most important antigens in this system are KEL1, or K, and the antithetical KEL2, or k. K and k are codominant autosomal alleles; approximately 9% of Whites and 2% of Blacks are K positive (ie, KK or Kk); the remainder are K negative (ie, kk). Antigens of the Kell blood group system are highly immunogenic and, excluding ABO, K are second only to RhD in its potential to elicit production of alloantibodies.

K antibodies are commonly found. Fortunately, because more than 90% of donor units are K negative, it is easy to obtain blood for transfusion to individuals with anti-K. In contrast, although anti-k is relatively rare, it is also of clinical significance, and only

**Table 8-1.** Overview of the Protein Blood Group Systems Other than Rh and LW

| ISBT Number | System Name   | Gene Name ISBT | Gene Name ISGN   | Red Cell Membrane Component                       | Number of Antigens* | Examples of Antigens                                                        |
|-------------|---------------|----------------|------------------|---------------------------------------------------|---------------------|-----------------------------------------------------------------------------|
| 002         | MNS           | <i>MNS</i>     | <i>GYP, GYPB</i> | Glycophorin A (CD235a) and glycophorin B (CD235b) | 46                  | M, N, S, s, U                                                               |
| 005         | Lutheran      | <i>LU</i>      | <i>LU</i>        | Lutheran glycoprotein (CD239)                     | 18                  | Lu <sup>a</sup> , Lu <sup>b</sup>                                           |
| 006         | Kell          | <i>KEL</i>     | <i>KEL</i>       | Kell glycoprotein (CD258)                         | 31                  | K, k, Js <sup>a</sup> , Js <sup>b</sup> , Kp <sup>a</sup> , Kp <sup>b</sup> |
| 008         | Duffy         | <i>FY</i>      | <i>DARC</i>      | Fy glycoprotein (chemokine receptor) (CD234)      | 6                   | Fy <sup>a</sup> , Fy <sup>b</sup>                                           |
| 009         | Kidd          | <i>JK</i>      | <i>SLC14A1</i>   | Kidd glycoprotein (urea transporter)              | 3                   | Jk <sup>a</sup> , Jk <sup>b</sup>                                           |
| 010         | Diego         | <i>DI</i>      | <i>SLC4A1</i>    | Band 3, anion exchanger 1 (CD233)                 | 20                  | Di <sup>a</sup> , Di <sup>b</sup> , Wr <sup>a</sup> , Wr <sup>b</sup>       |
| 011         | Cartwright    | <i>YT</i>      | <i>ACHE</i>      | Acetylcholinesterase                              | 2                   | Yt <sup>a</sup> , Yt <sup>b</sup>                                           |
| 012         | Xg            | <i>XG</i>      | <i>XG, MIC2</i>  | Xg <sup>a</sup> glycoprotein (CD99)               | 1                   | Xg <sup>a</sup>                                                             |
| 013         | Scianna       | <i>SC</i>      | <i>ERMAP</i>     | Sc glycoprotein (ERMAP)                           | 7                   | Sc1, Sc2, Sc3, Rd                                                           |
| 014         | Dombrock      | <i>DO</i>      | <i>ART4</i>      | Do glycoprotein (ART4)                            | 5                   | Do <sup>a</sup> , Do <sup>b</sup>                                           |
| 015         | Colton        | <i>CO</i>      | <i>AQP1</i>      | Aquaporin-1                                       | 3                   | Co <sup>a</sup> , Co <sup>b</sup>                                           |
| 017         | Chido/Rodgers | <i>CH/RG</i>   | <i>C4A, C4B</i>  | Complement component C4A/C4B                      | 9                   | Ch1, Ch2, Ch3, Rg <sup>1</sup> , Rg <sup>2</sup>                            |
| 019         | Kx            | <i>XK</i>      | <i>XK</i>        | Xk glycoprotein                                   | 1                   | Kx                                                                          |
| 020         | Gerbich       | <i>GE</i>      | <i>GYPC</i>      | Glycophorin C (CD236) and glycophorin D           | 7                   | Ge2, Ge3, Ge4                                                               |
| 021         | Cromer        | <i>CROM</i>    | <i>CD55</i>      | Decay accelerating factor (CD55)                  | 15                  | Cr <sup>a</sup>                                                             |
| 022         | Knops         | <i>KN</i>      | <i>CR1</i>       | Complement receptor 1 (CD35)                      | 9                   | Kn <sup>a</sup>                                                             |
| 023         | Indian        | <i>IN</i>      | <i>CD44</i>      | Hermes antigen (CD44)                             | 4                   | In <sup>a</sup> , In <sup>b</sup>                                           |
| 024         | OK            | <i>OK</i>      | <i>BSG</i>       | Neurothelin, basoglin (CD147)                     | 1                   | Ok <sup>a</sup>                                                             |
| 025         | RAPH          | <i>RAPH</i>    | <i>MER2</i>      | Unknown                                           | 1                   | MER2                                                                        |
| 026         | JMH           | <i>JMH</i>     | <i>SEMA7A</i>    | Semaphorin 7A (CD108)                             | 5                   | JMH1                                                                        |
| 029         | GIL           | <i>GIL</i>     | <i>AQP3</i>      | Aquaporin 3                                       | 1                   | GIL                                                                         |

\*Based on the ISBT Cape Town report.<sup>1</sup>

ISBT = International Society of Blood Transfusion; ISGN = International Society for Gene Nomenclature.

1 in 500 random-donor units is antigen negative. The other two sets of well-defined antithetical antigens are Kp<sup>a</sup>/Kp<sup>b</sup> and Js<sup>a</sup>/Js<sup>b</sup> (Fig 8-1).

Mothers with anti-K are relatively rare, but since the introduction of Rh prophylaxis, anti-K accounts for nearly 10% of cases of severe HDFN. In contrast to RhD, anti-K titers are not good predictors of fetal anemia. In addition, affected Kell-alloimmunized infants have lower reticulocyte counts and amniotic fluid bilirubin concentrations than RhD-sensitized infants. Anti-K has been hypothesized to suppress erythropoiesis at the progenitor cell level. It is important to determine if a fetus is at risk when the mother has anti-K. The prospective father should be typed and if he carries the K antigen, genotyping from amniocentesis can be performed using molecular techniques.

### Kell Variants

There are two rare but clinically interesting variants in the Kell blood group system. Rare individuals have red cells that completely lack the common Kell antigens. Although these cells exhibit the null phenotype (K<sub>0</sub>), they are morphologically normal and survive normally in vivo. In contrast, individuals lacking

the Kx antigen exhibit depressed levels of the Kell protein.<sup>7,8</sup> This phenotype, known as the McLeod syndrome, is associated with acanthocytic red cells and a mild chronic hemolytic anemia. The frequently reported neuromuscular symptoms in persons with McLeod phenotype may be caused by lack of Kx expression. Association of the McLeod phenotype with other rare syndromes, most frequently with CGD and DMD or Becker muscular dystrophy (BMD) is caused by large gene deletions encompassing *XK* together with adjacent genes *CYBB* that encodes a large subunit of cytochrome b<sub>558</sub> and is associated with X-linked CGD, and *DMD* that encodes dystrophin.<sup>15,16</sup>

## Duffy Blood Group System (ISBT 008)

### Structure and Function of the Duffy Protein

The Duffy gene encodes a glycoprotein of 336 amino acids with a molecular weight of 36kD. The Duffy glycoprotein has seven transmembrane  $\alpha$ -helical domains. The N-terminus is ectoplasmic and the C-terminus is intracellular (see Fig 8-2). Duffy protein functions as a chemokine receptor and is frequently

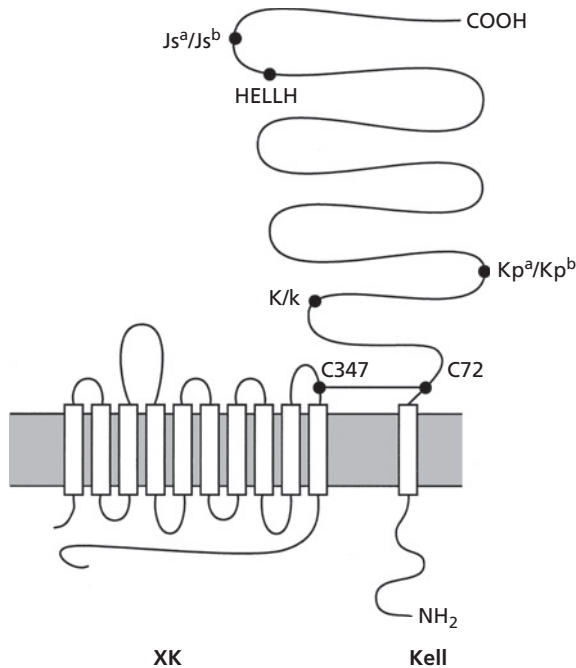


**Figure 8-1.** Schematic representation of the Kell/XK complex in the red cell membrane. The XK protein is a membrane protein with 10  $\alpha$ -helical transmembrane segments, while Kell has only one transmembrane domain, most of which is exposed on the ectoplasmic side. Due to multiple disulfide bonds, the ectoplasmic portion of Kell is a globular structure; however, it is represented here schematically so that the positions of the main alleles can be shown. A disulfide bond between Cys72 of Kell and Cys347 of XK connects the two proteins. The position of the pentameric sequence HELLH is shown. Sequences HEXXH are involved in zinc binding and catalytic activity of zinc endopeptidases. The K/k polymorphism at amino acid 193 changes the consensus sequence for N-glycosylation at Asn191, which is not glycosylated in K. This difference in glycosylation may be important for the marked antigenicity of K. Positions of two additional sets of antithetical antigens Kp<sup>a</sup>/Kp<sup>b</sup> and Js<sup>a</sup>/Js<sup>b</sup> at amino acids 281 and 597 are indicated.

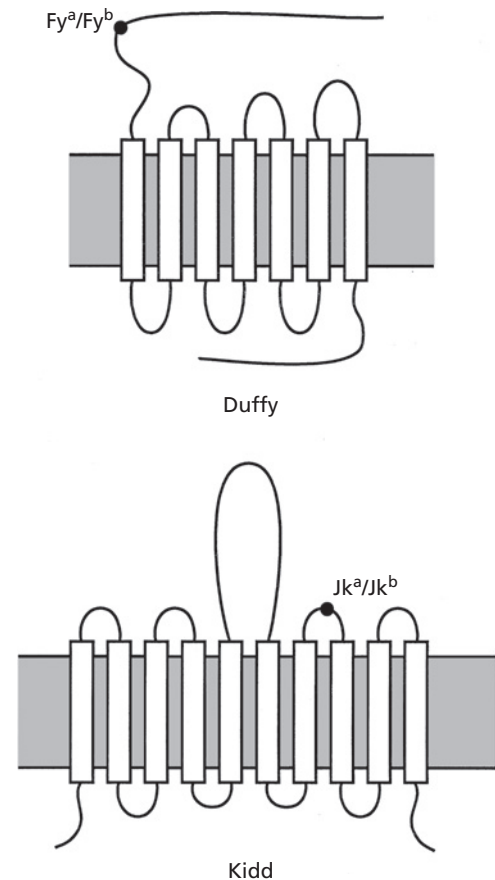


**Figure 8-2.** Schematic depiction of proteins carrying the Duffy and Kidd blood group antigens. The Duffy antigen receptor for chemokines (DARC) consists of seven transmembrane segments. The N-terminus is located extracellularly, functions as both the chemokine and *P. vivax* attachment site, and carries the Fy<sup>a</sup>/Fy<sup>b</sup> polymorphism. Kidd protein has 10 transmembrane segments, both N- and C-termini are intracellular and the amino acid determining the Jk<sup>a</sup>/Jk<sup>b</sup> polymorphism is located in the fourth ectoplasmic loop.

called the Duffy antigen receptor for chemokines (DARC).<sup>17,18</sup> Duffy binds both CXC chemokines, such as interleukin-8 (IL-8) and melanocyte growth-stimulating activity (MGSA), as well as CC chemokines, such as regulated on activation, normal T-cell expressed and secreted (RANTES) and macrophage chemoattractant protein (MCP-1).<sup>17</sup> It is currently not known why a red cell, with its limited metabolism and response to chemokine binding, would carry a significant number of chemokine receptors at its surface. One possible explanation is that the chemokine receptor acts as a scavenger for locally released chemokines. However, individuals who do not express the Duffy protein either on erythrocytes or in all tissues are phenotypically normal, suggesting that the Duffy protein is dispensable.

Duffy also functions as a receptor for the human malarial parasite *Plasmodium vivax*, which infects the erythrocytes of Duffy-positive individuals.<sup>19</sup> The binding site for *P. vivax* is located in the N-terminal ectoplasmic domain of Duffy. *P. vivax*

merozoites invade primarily reticulocytes, although reticulocytes and mature erythrocytes do not substantially differ in Duffy expression. This suggests that an additional receptor has to play a role in erythrocyte invasion.

### Duffy in Transfusion Medicine

The two main alleles of the Duffy blood group system are the antithetical codominant antigens Fy<sup>a</sup> and Fy<sup>b</sup> whose genetic determinant is a Gly/Asp polymorphism in position 44 (Fig 8-2).<sup>20</sup> These two alleles occur with similar frequencies in persons of European ancestry, Fy<sup>a</sup> being somewhat more common. The Fy(a-b-) phenotype is extremely rare among Whites but approximately two-thirds of African Americans and more than 90% of native West Africans are Fy(a-b-). This is most likely caused by the genetic adaptation for resistance to *P. vivax* malaria. However, it is not clear why this genetic advantage would lead almost to fixation of the Fy(a-b-) phenotype

in the indigenous population of West Africa. In contrast to the other malarial parasites, *P. vivax* causes a relatively mild form of malaria.<sup>17</sup>

A long-standing conundrum of immunohematology has been the well-known fact that transfused Fy(a-b-) people of African ancestry never develop anti-Fy<sup>b</sup>. This mystery was solved by the characterization of the mutation causing the Fy(a-b-) phenotype in native West Africans. The underlying mutation is a T→C substitution in the GATA site of the promoter region.<sup>21</sup> This substitution prevents binding of the erythroid transcription factor GATA-1 and abolishes transcription of the gene in the erythroid cells, while leaving the transcription of the *FY* gene in other tissues unaffected. Because the mutation occurred in the *FY*\*B allele, the Fy<sup>b</sup> antigen remains expressed in certain endothelial, epithelial, and brain cells, and, consequently, the transfusion recipient does not form antibodies against Fy<sup>b</sup>.

Fy<sup>a</sup> antibodies are found relatively frequently, constituting 6% to 10% of the clinically significant antibodies identified by immunohematology laboratories. For reasons not well understood, Fy<sup>b</sup> is a relatively poor immunogen and, consequently, anti-Fy<sup>b</sup> is considerably less common. Both immediate and delayed HTRs caused by Fy<sup>a</sup> incompatibility have been described, ranging from mild to severe hemolysis. HDFN is usually mild; only a few cases of severe HDFN have been reported. In contrast, anti-Fy<sup>b</sup> is associated only rarely with cases of mild HDFN and is usually found in delayed HTRs, although on rare occasions it has caused severe acute hemolysis.

## Kidd Blood Group System (ISBT 009)

The two main antigens of the Kidd blood group system, Jk<sup>a</sup> and Jk<sup>b</sup>, are found with almost identical frequencies in White populations. Jk<sup>a</sup> is a better immunogen and anti-Jk<sup>a</sup> is found more frequently than anti-Jk<sup>b</sup>. Anti-Jk<sup>a</sup> may cause severe immediate or delayed HTRs and, occasionally, HDFN. It is one of the most dangerous immune antibodies because of its tendency to decrease to undetectable levels in between transfusions and its relatively low affinity for Jk<sup>a</sup>-positive red cells. For these reasons, it accounts for a large proportion of delayed HTRs. Anti-Jk<sup>b</sup> may also cause immediate or delayed HTRs, albeit less severe than those caused by anti-Jk<sup>a</sup>. Several cases of mild HDFN caused by anti-Jk<sup>b</sup> have been reported.

The finding that cells of the Jk(a-b-) phenotype are resistant to lysis by 2M urea led to the discovery of the function of the protein carrying the Kidd antigens. Based on this finding and on in-vitro expression of the cloned Kidd cDNA,<sup>22-24</sup> it is now known that the protein is a urea transporter, although the importance of urea transport for erythrocytes is not completely understood. Its presence or absence may not be critical for red cell structure and function, because carriers of the Jk(a-b-) phenotype have red cells indistinguishable from those of controls.

Kidd is an integral protein with 10 transmembrane domains and both N- and C-termini located intracellularly (Fig 8-2). Of the five ectoplasmic loops, the longest, third loop is

N-glycosylated, and the relatively short fourth loop carries an Asp280Asn polymorphism corresponding to the Jk<sup>a</sup>/Jk<sup>b</sup> antigens.<sup>25</sup> Kidd protein is expressed not only on the red cell surface but also on neutrophils and in the kidney.

## MNS Blood Group System (ISBT 002)

### Structure and Function of Glycophorins A and B

Antigens of the MNS blood group system are carried by glycophorin A (GPA) and glycophorin B (GPB). Both molecules are present in a very high copy number in the plasma membrane;  $0.5-1.0 \times 10^6$  copies of glycophorin A and  $1-3 \times 10^5$  molecules of glycophorin B. Glycophorins A and B are encoded by homologous genes at chromosome 4q28-q31 that undoubtedly arose by gene duplication. Both glycoproteins are integral membrane proteins with a single transmembrane  $\alpha$ -helical segment and with the N-termini located extracellularly (Fig 8-3). Glycophorin A was the first protein whose primary structure was determined by amino acid sequencing.<sup>26</sup>

GPA carries the M and N antigens. The antigenic difference is caused by substitutions in position 1 and 5. Serine in position 1 and glycine in position 5 correspond to the M antigen, while the N allele has leucine and glutamic acid in these positions (Fig 8-3 and Table 8-2). An important requirement for recognition of these antigens by human antibodies is that serines in positions 2 and 4 and threonine in position 3 are O-glycosylated.

GPB is homologous with GPA. Although the genes are greater than 95% identical, *GYPB* encodes a shorter protein because a point mutation at the 5' splicing site of the third intron prevents incorporation of exon 3 into the translated mRNA. Because *GYPB* arose by duplication of the N allele of *GYPA*, *GYPA*\*N, and the first 26 amino acids of GPB are therefore identical to those of GPA with N specificity, GPB expresses an N-like antigen designated as 'N' (Table 8-2 and Fig 8-3). The S and s alleles of GPB differ at amino acid position 29. The S allele contains methionine and the s allele, threonine. GPB also carries the U antigen whose epitope is adjacent to the point where GPB enters into the lipid bilayer (Fig 8-3).

As is frequently the case with genes arising by duplication and located next to each other, unequal crossing over or gene conversion may easily occur. Consequently, numerous hybrid molecules containing portions of GPA and GPB have been described. This phenomenon is partly responsible for the 46 currently known antigens of the MNS blood group system, particularly for many of the Miltenberger (Mi) variants.

GPA associates in the red cell membrane with the band 3 protein. The epitope of the Wr<sup>b</sup> antigen from the Diego blood group system (see below) is formed by both GPA and band 3. This clearly demonstrates the intimate association of GPA and band 3 in the plasma membrane.<sup>27</sup>

### MNS in Transfusion Medicine

The most commonly encountered antibodies are directed against the M, N, S, and s antigens. Anti-M is often found in the sera

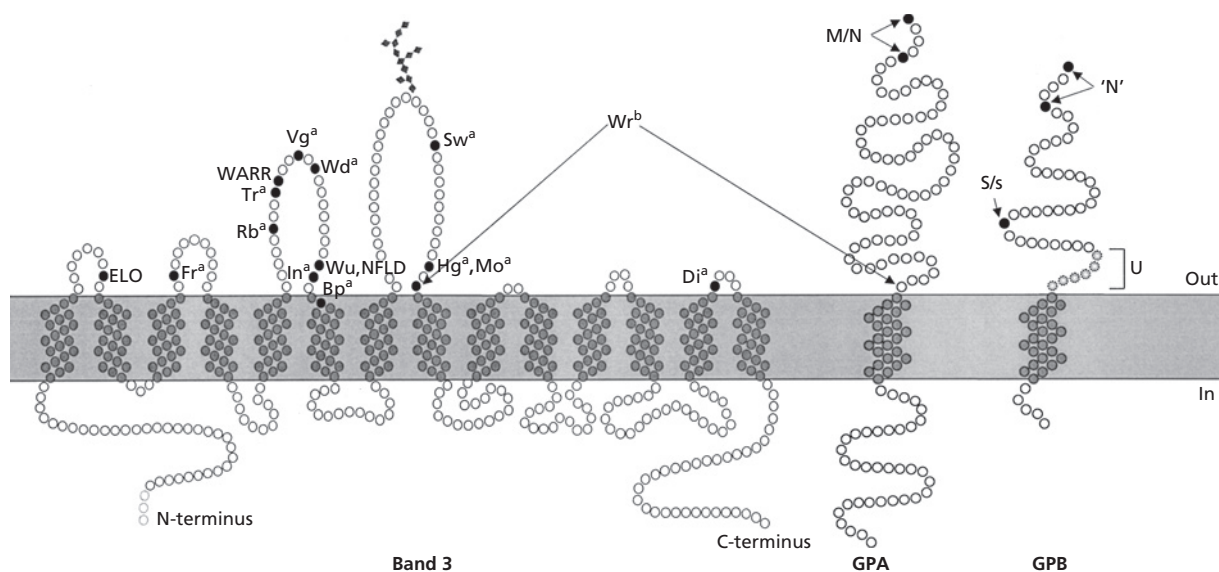


**Figure 8-3.** Antigens of the Diego and MNS blood group systems in a scheme of band 3 and of glycophorins A and B. The membrane domain of band 3 with 14 transmembrane segments is shown. Mutations underlying the Diego blood group antigens are located in the putative first, second, third, fourth, and seventh ectoplasmic loops. Positions of the M and N antigens in GPA and of the 'N', S, s, and U antigens in GPB are indicated. Arrows point to the sites in band 3 and GPA that are involved in formation of the  $Wr^b$  epitope and, therefore, have to come into close contact in the membrane.

**Table 8-2.** N-Terminal Sequences of the M and N Alleles of Glycophorin A (GPA) and of the S and s Alleles of Glycophorin B (GPB)

| Antigen | Amino Acid Sequence                                                  |
|---------|----------------------------------------------------------------------|
|         | 1 5 26 29                                                            |
|         | ↓ ↓ ↓ ↓                                                              |
| M (GPA) | SSTTG <del>V</del> AMHTSTSSSVTKSYISSQTNDTHKRD <del>T</del> YAATPRA.. |
| N (GPA) | LSTTE <del>V</del> AMHTSTSSSVTKSYISSQTNDTHKRD <del>T</del> YAATPRA.. |
| S (GPB) | LSTTE <del>V</del> AMHTSTSSSVTKSYISSQTNGEMGQLVHRFTVPA..              |
| s (GPB) | LSTTE <del>V</del> AMHTSTSSSVTKSYISSQTNGETGQLVHRFTVPA..              |
|         | <---> ↑                                                              |
|         | M/N; 'N' S/s                                                         |

Differences between M and N reside in amino acids 1 and 5. First 26 amino acids of GPB are identical to GPA and GPB therefore expresses an N-like antigen 'N'. The differences between the S and s antigens correspond to a M/T polymorphism in position 29 of GPB. S = serine; L = leucine; G = glycine; E = glutamic acid; M = methionine; T = threonine.

of persons who have not been exposed to human red cells. M antibodies are mostly IgM; however, they frequently contain an IgG component and, occasionally, are exclusively IgG. Anti-M is rarely clinically significant; hemolytic anti-M is usually IgG and reactive at 37°C. Anti-N is rare, most likely because of the immune tolerance induced by the 'N' antigen on GPB. Strong and potentially clinically significant antibodies have been observed in persons of the rare phenotype M+N-S-s-U—who do not express GPB (hence 'N'). In contrast to anti-M and anti-N, antibodies to S, s, and U usually occur after exposure to

**Table 8-3.** Comparison of the Most Frequently Encountered Antibodies in the MNS System

| Anti-M and -N            | Anti-S, -s, and -U     |
|--------------------------|------------------------|
| Naturally occurring      | Exposure is required   |
| Cold IgM                 | Warm IgG               |
| Dosage                   | Minimal dosage         |
| Clinically insignificant | Clinically significant |

allogeneic red cells. All are capable of causing HTRs and HDFN. The most important antibodies of the MNS system are compared in Table 8-3.

## Diego Blood Group System (ISBT 010)

Antigens of the Diego blood group system are carried by erythrocyte band 3 protein (anion exchanger 1), the most abundant integral protein of the red cell membrane together with GPA (see above). Band 3 is also one of the most important proteins for the structure and function of the membrane because it maintains red cell integrity by linking the red cell membrane to the underlying spectrin-based membrane skeleton. It also mediates exchange of chloride and bicarbonate anions across the plasma membrane, thereby significantly increasing the carrying capacity of blood for carbon dioxide.

Band 3 consists of a cytoplasmic and a membrane domain. The membrane domain contains 14 transmembrane helices

connected by ecto- and endoplasmic loops<sup>28,29</sup> (Fig 8-3). The fourth loop of band 3 is N-glycosylated and the attached carbohydrate chain carries over half of the red cell ABO blood group epitopes.<sup>30</sup> Several disorders of red cell structure and function have been associated with mutations in the band 3 gene, including Southeast Asian ovalocytosis,<sup>31</sup> autosomal dominant spherocytosis,<sup>32-34</sup> and distal renal tubular acidosis.<sup>35</sup>

Despite being the most abundant protein of the red cell membrane, band 3 until recently has not been known to carry any red cell antigens with the exception of the ABO antigens residing on the attached carbohydrate chains. It was only in 1992 that Spring et al<sup>36</sup> reported that the Memphis II variant of erythroid band 3 protein carries the Di<sup>a</sup> blood group antigen. Di<sup>a</sup> was originally described in South American Indians by Layrisse et al in 1955.<sup>37</sup> The antithetical antigen Di<sup>b</sup> was reported by Thompson et al in 1967.<sup>38</sup> Di<sup>a</sup> and Di<sup>b</sup> represent codominantly expressed gene products. Di<sup>a</sup> is a low-incidence blood group antigen in persons of European ancestry who carry the antithetical high-incidence antigen Di<sup>b</sup>. Prevalence of Di<sup>a</sup> is as high as 8% in certain areas of Southeast Asia and reaches up to 40% in some groups of South American Indians.<sup>37</sup> Di<sup>a</sup> was used as one of the markers for studying migration of people from Southeast Asia across the Bering Strait and southward through the American continents. Cloning and sequencing of band 3 gene identified the substitution 854 Pro→Leu in the last ectoplasmic loop of band 3 as the molecular basis of the Di<sup>a</sup> antigen. Di<sup>b</sup> corresponds to the wild type band 3 with proline in position 854.<sup>39</sup>

Subsequently, the low-incidence blood group antigen Wr<sup>a</sup> was mapped to the fourth ectoplasmic loop.<sup>27</sup> The antithetical Wr<sup>b</sup> antigen is seen only when both GPA and band 3 protein are expressed in the erythrocyte membrane, which helped to characterize the site of interaction between band 3 and GPA (Fig 8-3). During the last several years, numerous additional low-incidence antigens have been associated with single point mutations on band 3 and included in the Diego system.<sup>40,41</sup> Positions of the mutated amino acids in the band 3 molecule are shown in Fig 8-3, which also indicates the regions of band 3 and glycophorin A that interact in the membrane and are involved in formation of the Wr<sup>b</sup> antigen.

Some antigens of the Diego blood group system have been localized to the regions of band 3 protein that have been implicated in the adhesion of abnormal red cells, such as sickle cells or malaria-infected erythrocytes, to vascular endothelium.<sup>42,43</sup> Erythrocytes from carriers of low-incidence blood group antigens in ectoplasmic loops of band 3 may serve as a model for evaluation of the sequence requirements for adhesion. The so-called “senescent” or “aging” red cell antigen may also be located in the ectoplasmic loops of band 3.

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### Gerbich Blood Group System (ISBT 020)

As in the MNS system, antigens of the Gerbich system are located on glycophorins C and D. The glycophorin terminology is in fact

the only common feature of these two classes of glycophorins. There is otherwise no homology between the Gerbich and MNS genes. Unlike GPA and GPB, GPC and GPD are the products of a single gene and, unlike *GYP A* and *GYP B*, this gene is expressed in multiple tissues. The considerably less abundant and smaller GPD is produced from a shorter transcript than GPC. Although present in much smaller copy numbers than GPA and GPB, GPC plays an important role in the structural integrity of the red cell membrane. In the Leach phenotype, deletions of exons 3 and 4 or a frameshift mutation lead to complete absence of glycophorins C and D from the plasma membrane. The affected individuals have moderate elliptocytosis and decreased red cell deformability and mechanical stability. Antibodies in the Gerbich system are rare and, in the vast majority of cases, clinically insignificant.

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### Colton and GIL Blood Group Systems (ISBT 015 and 029)

Antigens of these two blood group systems are carried by members of the large aquaporin family. Antibodies against the two antigens of the Colton blood group system, the high-frequency Co<sup>a</sup> and the less common, antithetical Co<sup>b</sup>, are rare and have only rarely been associated with mild HTRs and mild HDFN. These two antigens, and the Co3 antigen that is present on all red cells except those of the very rare null phenotype Co(a-b-), are carried by aquaporin-1,<sup>44</sup> a member of a large family of water channels.<sup>45</sup> It is present in the membrane as a tetramer. Expression of aquaporin-1 in *Xenopus* oocytes is associated with dramatic swelling and lysis of the cell.<sup>46</sup> At the same time, the Co(a-b-) phenotype is associated with only slightly abnormal red cells and with normal kidney function despite the fact that aquaporin-1 is the major water channel of human kidney.<sup>47,48</sup> The GIL antigen is carried by aquaporin-3, which differs from aquaporin-1 in that it transports glycerol, water, and urea.<sup>49</sup>

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### Lutheran Blood Group System (ISBT 005)

The Lutheran blood group system contains multiple antigens; however, clinically significant antibodies are rarely encountered. The most important antigens are Lu<sup>a</sup> and Lu<sup>b</sup>. The frequency of Lu<sup>a</sup> is less than 10% in most populations, while Lu<sup>b</sup> is a high-frequency antigen with an average prevalence of 99.8% in all populations. Lutheran antigens are poorly developed at birth and, not surprisingly, anti-Lu<sup>a</sup> has been associated only rarely with mild cases of HDFN. It does not cause transfusion reactions. Lu<sup>b</sup> is somewhat more immunogenic and anti-Lu<sup>b</sup> has caused mild or moderate HTRs and mild HDFN. Of historical note, Lu and Se (Chapter 6) were the first two loci for which an autosomal linkage in humans was demonstrated.

Lutheran antigens reside on B-CAM/LU (Fig 8-4), a pair of spliceosomes (protein products arising from the same gene because of alternative splicing of hnRNA) that belong to the



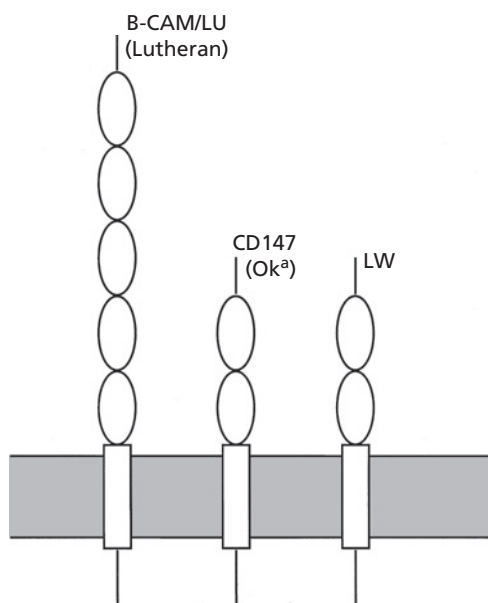


**Figure 8-4.** Members of the immunoglobulin superfamily carrying blood group antigens. B-CAM/LU contains five immunoglobulin domains and carries antigens of the Lutheran blood group system. CD147, or neurothelin, contains two Ig domains and carries the Ok<sup>a</sup> antigen. CD147 is similar to the LW protein, which was discussed in Chapter 7.

immunoglobulin superfamily.<sup>50,51</sup> Basal cell adhesion molecule (B-CAM) is involved in adhesion of the basal surface of epithelial cells to the basement membrane. B-CAM/LU is a receptor for laminin.<sup>52</sup> Expression of B-CAM/LU is increased on red cells from patients with sickle cell disease<sup>52</sup> and on a number of malignant epithelial tumors, which also lose the polarity of B-CAM/LU expression found in normal tissues.<sup>51</sup>

The null phenotype, Lu(a–b–), is rare but quite interesting. It may result from three different patterns of inheritance. A recessive pattern of inheritance is associated with an exceedingly rare null allele. In the most common dominant type of inheritance, the expression of Lu can be suppressed by a single copy of the suppressor gene *In(Lu)*. In addition to reducing the expression of Lutheran antigens to levels undetectable by standard serologic techniques, *In(Lu)* also decreases the expression of antigens P1, i, In<sup>a</sup> and In<sup>b</sup> (see below), and AnWj, a receptor for *Haemophilus influenzae*. The third cause of the Lu(a–b–) phenotype is the presence of an X-linked recessive suppressor gene *XS2*.<sup>53</sup>

### Indian Blood Group System (ISBT 023)

The four antigens of this system, In<sup>a</sup>, In<sup>b</sup>, INFI, and INJA reside on CD44, an adhesion molecule expressed in leukocytes, fibroblasts, epithelial cells, and other tissues. CD44 is an important lymphocyte marker that functions as a hyaluronan receptor,<sup>54</sup> and a lymphocyte homing receptor (Fig 8-4). Transfection of

nonadherent cell lines with CD44 cDNA confers an adherent phenotype.<sup>55</sup> As with the Lutheran antigens, expression of In<sup>a</sup> and In<sup>b</sup> is suppressed by *In(Lu)*.

### Xg Blood Group System (ISBT 012)

The only antigen of the Xg system, Xg<sup>a</sup>, is carried by a glycoprotein of 180 amino acids.<sup>56</sup> The function of Xg glycoprotein in erythrocytes is not known. Designation of this blood group system reflects the fact that the *PBDX* gene,<sup>57</sup> which encodes Xg, is located on the X-chromosome. Xg is 48% homologous to CD99, an adhesion molecule. Xg<sup>a</sup> antibodies are clinically insignificant.

### Scianna Blood Group System (ISBT 013)

The seven antigens of the Scianna blood group system are carried by the erythrocyte membrane-associated protein (ERMAP), potentially a receptor/signal transduction molecule specific for erythroid cells.<sup>58</sup> Mild HDFN and mild postransfusion hemolysis caused by anti-Sc-2 and anti-Sc-3 antibodies have been reported.

### Chido/Rodgers Blood Group System (ISBT 017)

Antigens of the Chido/Rodgers blood group system are the only protein antigens that are not produced by red cells but instead adhere to the red cell surface (Lewis antigens are glycolipids). They are carried by the complement component C4. Although antibodies against the nine known antigens of the system are generally benign, a severe anaphylactic reaction following a transfusion of platelets to a patient with anti-Ch3 has been described.<sup>59</sup>

### Knops Blood Group System (ISBT 022)

Antigens are located on the C3b/C4b complement receptor 1 (CR1, CD35). CR1 protects erythrocytes from autohemolysis by inhibiting the classical and alternative complement pathways through cleavage of C4b and C3b. CR1 is a large 190- to 280-kD molecule. It contains 30 complement control protein domains (CCPD) of about 60 amino acids. Seven CCPDs form a long homologous repeat (LHR) of about 450 amino acids. Various forms of CR1 contain up to 6 LHRs. Erythrocyte CR1 binds immune complexes and carries them to the liver and spleen for removal. Expression of CR1 on erythrocytes varies widely from 20 to 1500 molecules and is decreased in hemolytic anemias, AIDS, systemic lupus erythematosus, and other autoimmune disorders. *Plasmodium falciparum*-infected erythrocytes deficient in CR1 have greatly reduced rosetting capacity, indicating an essential role for CR1 in rosette formation and raising the possibility that CR1 polymorphisms in Africans that influence the interaction between erythrocytes and parasite-encoded protein



PfEMP1 may protect against severe malaria.<sup>60</sup> CR1 could therefore be a potential target for future therapeutic interventions to treat severe malaria.<sup>61,62</sup>

### Blood Group Antigens on Phosphatidylinositol-Linked Proteins—Cartwright (ISBT 011), Dombrock (ISBT 014), Cromer (ISBT 021), and JMH (ISBT 026)

The common denominator of these antigens is the linkage of the carrier protein to the glycosylphosphatidylinositol (GPI) anchor (Fig 8-5). The Cartwright (Yt) blood group system consists of two antigens, Yt<sup>a</sup> and Yt<sup>b</sup>, which are located on red cell acetylcholinesterase. Most examples of anti-Yt<sup>a</sup> are benign.

The five antigens of the Dombrock system are located on a recently characterized glycoprotein.<sup>63</sup> Homology studies suggest that the Dombrock molecule is a member of the adenosine 5'-diphosphate (ADP)-ribosyltransferase ectoenzyme gene family. Dombrock expression is developmentally regulated during erythroid differentiation and occurs at highest levels in the fetal liver. Do<sup>a</sup> and Do<sup>b</sup> antigens differ in a single amino acid substitution within the RGD motif of the molecule.<sup>63,64</sup>

The Cromer blood group system contains 15 antigens located on decay accelerating factor (DAF, CD55), a complement regulatory protein. Although DAF is the first complement regulatory protein identified, it plays only a minor role in complement-mediated lysis, the more important being the MIRL (CD59) molecule. This was clearly demonstrated in the case of the null

phenotype, Inab, which is associated with lack of DAF expression on all circulating cells but not with increased hemolysis.<sup>65</sup>

The only antigen of the JMH blood group system resides on a GPI-linked protein semaphorin 7A (CD108) that is part of a plasma membrane complex associated with intracellular protein kinases.<sup>66</sup> CD108 is expressed in multiple tissues and may play a role in signal transduction. Semaphorin 7A is a surprisingly diverse molecule<sup>67</sup> and four new antigens have been recently added to the JMH blood group system.<sup>1</sup>

Antibodies in these four blood group systems have been associated only occasionally with mild HTRs or HDFN. Expression of all GPI-linked antigens is, not surprisingly, decreased in paroxysmal nocturnal hemoglobinuria, a disorder caused by defects in the X-linked phosphatidylinositol glycan class A (PIG-A) gene, which participates in an early step of GPI anchor synthesis.

### OK and RAPH Blood Group System (ISBT 024 and 025)

Each of these two blood group systems contains only one antigen. Ok<sup>a</sup> is a high-incidence antigen; rare Ok(a-) individuals have so far been reported only in Japan. Ok<sup>a</sup> is carried on an N-glycosylated protein with an apparent molecular weight of 35 to 69 kD, which is expressed in multiple tissues. The Ok glycoprotein is a member of the immunoglobulin superfamily. As with LW, its extracellular domain contains two immunoglobulin domains. The function of the Ok glycoprotein in erythrocytes is not known.

MER2 is the only antigen of the RAPH system. All MER2 antibodies so far reported have been found in patients on hemodialysis. The molecular basis of the antigen is not known.

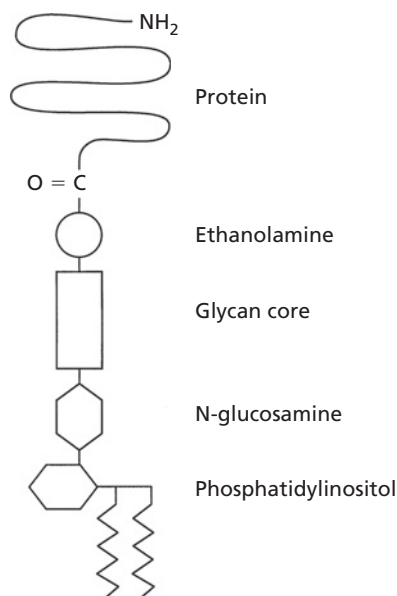


**Figure 8-5.** Schematic representation of a GPI-anchored protein. The membrane anchor is provided by phosphatidylinositol. The inositol moiety binds to a glycan core via a molecule of N-glucosamine. The glycan core is attached via ethanolamine to the C-terminus of the protein.

### Summary

The century-long history of modern transfusion medicine and immunohematology practice led to the characterization of an enormous number of blood group antigens with often confusing terminology. These antigens have been arranged into a complex framework of blood group systems, collections, and low- and high-frequency antigens. Fortunately, advances in biochemical and molecular biology techniques in the past two decades led to a detailed structural characterization of most proteins carrying blood group antigens and to a better understanding of the relation between gene variations, amino acid polymorphisms, protein structure, and immunogenicity of individual antigens. Better understanding of the molecular biology, biochemistry, and immunogenicity of proteins carrying blood group antigens will undoubtedly contribute to accurate compatibility testing and to safe transfusion of red cells.

### Disclaimer

The author has disclosed no conflicts of interest.

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# 9

## Anemia and Red Blood Cell Transfusion

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Red cell transfusion is an extremely common medical intervention. About 75 million units of Red Blood Cells (RBCs) are collected worldwide,<sup>1</sup> and in the United States, 15.8 million allogeneic units were donated in 2006.<sup>2</sup> In Northern England, about 50% of units are given to medical patients, and 40% to surgical patients; hip replacement and coronary artery bypass graft (CABG) surgery were the most common surgical indications.<sup>3</sup> RBC units are also frequently administered to critically ill patients, as a supportive therapy to patients receiving chemotherapy and marrow transplants, and to patients with blood loss from medical conditions such as gastrointestinal bleeding.<sup>4</sup>

This chapter reviews the current knowledge about red cell transfusion and the risk posed by anemia. The adaptive physiologic mechanisms in response to acute blood loss are described, as well as more chronic decreases in red cell mass. These discussions include a review of oxygen transport, adaptive mechanisms in anemia, and microcirculatory effects of anemia. Some of these fundamental principles are followed by a review of clinical outcomes in patients with anemia. Similarly, the clinical consequences (both risks and benefits) of red cell transfusion are outlined. This overview summarizes preclinical, observational, and controlled trials. Guidelines on transfusion practices are examined. The first three sections are based on systematic reviews of the literature. The fourth section describes an approach to decision making in red cell transfusion that is occasionally evidence-based, but frequently based on the authors' views.

### Physiologic Adaptations to Blood Loss and Anemia

#### Overview of Oxygen Transport

Hemoglobin is a complex molecule that consists of four globin moieties, each incorporating an iron-containing heme ring where

oxygen is bound according to its partial pressure ( $PO_2$ ). The oxygen-binding affinity of hemoglobin is graphically represented by a sinusoidal relationship between hemoglobin oxygen saturation and  $PO_2$ . This relationship, referred to as the *oxyhemoglobin dissociation curve*, enables both efficient loading in the lungs at high  $PO_2$  and efficient unloading in the tissues at low  $PO_2$  (Fig 9-1). However, the affinity of hemoglobin for oxygen (the degree to which oxygen molecules saturate the hemoglobin binding sites at a given  $PO_2$ ) is altered by various disease states and may play a significant adaptive role in the response to anemia. The amount of oxygen delivered, either to the whole body or to specific organs, is the product of blood flow and arterial oxygen content. For the whole body, oxygen delivery ( $DO_2$ ) is the product of total blood flow or cardiac output (CO) and arterial oxygen content ( $CaO_2$ ), represented by the following equation (#1):

$$DO_2 = CO \times CaO_2$$

When ambient air is breathed under normal conditions, the oxygen present in arterial blood is bound to hemoglobin. When fully saturated, 1 g of hemoglobin binds 1.39 mL of oxygen. In addition, a small amount of hemoglobin also is dissolved in plasma water. The negligible amount of dissolved oxygen is directly proportional to the partial pressure and is calculated by multiplying  $PO_2$  by a constant ( $k = 0.00301$  mL/mm Hg), called the *solubility coefficient*. Thus, under most circumstances, arterial oxygen content can be approximated from the portion bound to hemoglobin with the following equation (#2):

$$CaO_2 \text{ (mL/L)} = \%Sat \times 1.39 \text{ (mL/g)} \times Hb \text{ (g/dL)}$$

When  $CaO_2$  from equation 2 is substituted into equation 1, the results are as shown in the following equation (#3):

$$DO_2 = CO \times (\%Sat \times 1.39 \times Hb)$$

Where CO is cardiac output in liters per minute, %Sat is hemoglobin oxygen saturation in percent, and Hb is hemoglobin concentration in g/dL.

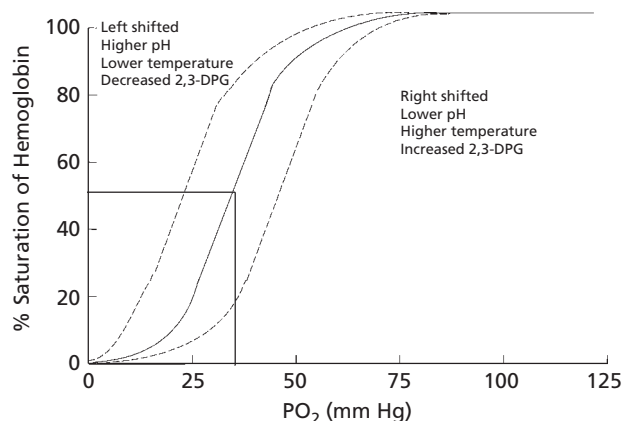


**Figure 9-1.** Oxyhemoglobin dissociation curve. The  $P_{50}$  is a measurement of the partial pressure of oxygen when hemoglobin is 50% saturated. The solid line represents oxygen-binding affinity to the hemoglobin molecule at standard temperature ( $37^{\circ}\text{C}$ ) and pH (7.4). The  $P_{50}$  of this curve is usually between 26 and 28 mm Hg. The dashed lines represent hypothetical shifts in the curve. A rightward shift represents a decreased affinity of hemoglobin for oxygen or an increase in  $P_{50}$ . It may be caused by increased levels of 2,3-diphosphoglycerate (2,3-DPG), temperature, or decreased pH. A shift to the left represents an increased affinity of oxygen for hemoglobin and is measured by a decrease in  $P_{50}$ . A leftward shift may result from decreased temperature, levels of 2,3-DPG or increased pH.

Tissue hypoxia and anoxia eventually occur if oxygen delivery decreases to a level at which it is no longer adequate to meet the metabolic demands of the tissues. From Equations 1 and 3, it is apparent that tissue hypoxia can be caused by decreased oxygen delivery resulting from decreases in either hemoglobin concentration (anemic hypoxia), cardiac output (stagnant hypoxia), or hemoglobin saturation (hypoxic hypoxia). Each of the determinants of  $\text{DO}_2$  has substantial physiologic reserves, thereby enabling the human body to adapt to significant increases in oxygen requirements or decreases in one of the determinants of  $\text{DO}_2$  as a result of various diseases.

In health, the amount of oxygen delivered to the whole body exceeds resting oxygen requirements twofold to fourfold. For example, with a hemoglobin level of 15 g/dL, a hemoglobin oxygen saturation of 99%, and a cardiac output of 5 L/minute, oxygen delivery is 1032 mL/minute. At rest, the amount of oxygen required or consumed by the whole body ranges from 200 to 300 mL/minute. A decrease in hemoglobin concentration to 10 g/dL would result in an oxygen delivery of 688 mL/minute. Despite this 33% decrease in oxygen delivery, there remains a twofold excess of oxygen delivered compared with oxygen consumed. However, a further decrease in hemoglobin concentration to 5 g/dL with all other parameters (including cardiac output) remaining constant, decreases oxygen delivery to a critical level of 342 mL/minute. Under stable experimental conditions, this dramatic decrease in oxygen delivery still exceeds oxygen consumption. However, at the critical level or threshold of oxygen— $\text{DO}_2(\text{crit})$ —oxygen delivery equals oxygen consumption. Further

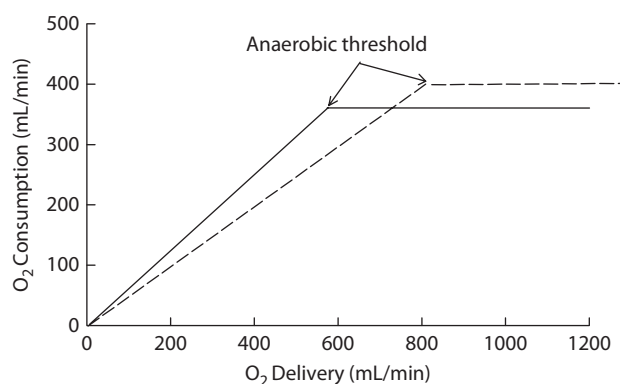


**Figure 9-2.** Relationship between oxygen consumption and oxygen delivery. The solid line shows the biphasic consumption and delivery. The dashed line illustrates the postulated changes in the relation with diseases such as sepsis and acute respiratory distress syndrome. The anaerobic threshold is shifted to the right, suggesting that patients need increased levels of delivery to avoid ongoing ischemic damage to vital organs.

decreases in hemoglobin concentration result in inadequate oxygen delivery to tissues. There is, therefore, a biphasic relationship between oxygen delivery and consumption (Fig 9-2). One phase is an oxygen delivery-independent portion, in which oxygen consumption is independent of oxygen delivery above the  $\text{DO}_2(\text{crit})$  delivery threshold. The other is a delivery or supply-dependent portion, in which oxygen delivery is directly related to oxygen consumption. The latter portion of this relationship below the  $\text{DO}_2(\text{crit})$  indicates the presence of tissue hypoxia. Both laboratory and clinical studies have attempted to determine the  $\text{DO}_2(\text{crit})$ . In the most rigorous clinical study,<sup>5</sup> the investigators found a threshold value of 4 mL/minute/kg. In other clinical and laboratory studies, investigators found values in the range of 6 to 10 mL/minute/kg.<sup>5,6</sup> The  $\text{DO}_2(\text{crit})$ , or the anaerobic threshold, is not a single, fixed value but varies substantially depending on factors such as basal metabolic rate, the specific organ or tissue, some disease states, and perhaps such complex factors as patient age or genetic composition. In the previous example, cardiac output did not increase as would otherwise be expected in anemia.

Once it is oxygenated, blood is distributed to all organs and tissues through the arterial tree into the microcirculation. Organ blood flow is controlled by arterial tone in medium-sized vessels and is primarily responsive to changes in autonomic stimulation and the release of locally generated vasodilating substances. Within organ systems, red cells are carried into the microcirculation, where oxygen is released to the tissues through a thin-walled capillary network. Once released, oxygen diffuses through the interstitial space, finally finding its way into the cell and its mitochondria to be used in cellular respiration. Each of these physiologic mechanisms can be altered in disease, as described later. Additional adaptive changes in the microcirculation enhance oxygen delivery in anemia.<sup>7</sup>

### Adaptive Mechanisms in Anemia

In anemia, oxygen-carrying capacity decreases, but tissue oxygenation is preserved at hemoglobin levels well below 10 g/dL (Table 9-1). After the development of anemia, adaptive changes include a shift in the oxyhemoglobin dissociation curve, hemodynamic alterations, and microcirculatory alterations. The shift to the right of the oxyhemoglobin dissociation curve in anemia is primarily the result of increased synthesis of 2,3-diphosphoglycerate (2,3-DPG) in red cells.<sup>8-10</sup> This rightward shift enables more oxygen to be released to the tissues at a given  $PO_2$ , offsetting the effect of reduced oxygen-carrying capacity of the blood. In-vitro studies have shown rightward shifts in the oxyhemoglobin dissociation curve with increases in temperature and decreases in pH.<sup>11</sup> Although clinically important shifts have been documented in a number of studies, hemoglobin oxygen saturation generally is measured in arterial specimens processed at standard temperature and pH. Therefore, current measurement techniques do not reflect oxygen-binding affinity or unloading conditions in the patient's microcirculatory environment, which may be affected by temperature, pH, and a number of disease processes. The shift in the oxyhemoglobin dissociation curve caused by decreases in pH (increase in hydrogen ion concentration) is the Bohr effect.<sup>11,12</sup> Because changes in pH rapidly affect the ability of hemoglobin to bind oxygen, this mechanism has been postulated to be an important early adaptive response to anemia.<sup>13</sup> However, the equations describing the physical process indicate that a very large change in pH is needed to modify the partial pressure of oxygen at which hemoglobin is 50% saturated

with oxygen ( $P_{50}$ ) by a clinically important amount ( $\sim 10$  mm Hg). As a result, the Bohr effect is unlikely to have significant clinical consequences.<sup>11,12</sup>

Several hemodynamic alterations occur after the development of anemia. The most important determinant of cardiovascular response is the patient's volume status, or more specifically left ventricular preload. The combined effect of hypovolemia and anemia often occurs as a result of acute blood loss. Acute anemia can cause tissue hypoxia or anoxia through both diminished cardiac output (stagnant hypoxia) and decreased oxygen-carrying capacity (anemic hypoxia).<sup>14</sup> The body primarily attempts to preserve oxygen delivery to vital organs by compensatory increases in myocardial contractility and heart rate as well as increased arterial and venous vascular tone mediated through increased sympathetic discharge. In addition, a variety of mechanisms redistribute organ blood flow. The adrenergic system plays an important role in altering blood flow to and within specific organs. The renin-angiotensin-aldosterone system is stimulated to retain both water and sodium. Losses ranging from 5% to 15% in blood volume result in variable increases in resting heart rate and diastolic blood pressure. Orthostatic hypotension often is a sensitive indicator of relatively small losses in blood volume not sufficient to cause a marked decrease in blood pressure. Larger losses result in progressive increases in heart rate and decreases in arterial blood pressure accompanied by evidence of organ hypoperfusion. The increased sympathetic tone diverts an ever decreasing global blood flow (cardiac output) away from the splanchnic, skeletal, and cutaneous circulation

**Table 9-1.** Physiologic Changes Associated with Anemia

#### Oxyhemoglobin dissociation curve

- Anemia shifts the oxyhemoglobin curve to the right because of increased 2, 3-biphosphoglycerate levels.
- Anemia causes clinically significant rightward shifts in the oxyhemoglobin curve because of the Bohr effect.
- The shift in the oxyhemoglobin curve has been clearly established in many forms of anemia (excluding hemoglobinopathies).
- The shift in the oxyhemoglobin curve has been clearly established in a number of human diseases.

#### Cardiac output

- Cardiac output increases with increasing degrees of normovolemic anemia.
- Increased cardiac output in normovolemic anemia is a result of increased stroke volume.
- The contribution of increased heart rate to the increase in cardiac output following normovolemic anemia is variable.

#### Other hemodynamic alteration

- Changes in blood viscosity result in many of the hemodynamic changes in normovolemic anemia.
- Normovolemic anemia is accompanied by increased sympathetic activity.
- Normovolemic anemia causes increased myocardial contractility.
- Normovolemic anemia causes a decrease in systemic vascular resistance.
- Normovolemic anemia results in a redistribution of cardiac output toward the heart and brain and away from the splanchnic circulation.
- Maximal global oxygen delivery occurs at hemoglobin values of 10 g/dL to 11 g/dL.
- Global oxygen delivery declines above and below hemoglobin values of 10 g/dL to 16 g/dL.

#### Coronary and cerebral blood flow

- Coronary blood flow is increased during anemia.
- Cerebral blood flow is increased during anemia.
- Coronary artery disease in the presence of moderate degrees of anemia (hemoglobin values below 9 g/dL) results in impaired left ventricular contractility or ischemia.
- Moderate anemia does not aggravate cerebral ischemia in patients with cerebrovascular disease.

toward the coronary and cerebral circulation. Once vital organ systems such as the kidneys, the central nervous system, and the heart are affected, the patient is considered in hypovolemic shock. Although the American College of Surgeons Committee on Trauma<sup>15</sup> has categorized the cardiovascular and systemic response to acute blood loss according to degree of blood loss, many of these responses are modified by the rapidity of blood loss and patient characteristics, such as age, coexisting illnesses, preexisting volume status, hemoglobin value, and the use of medications having cardiac effects ( $\beta$ -blockers) or peripheral vascular effects (antihypertensives).

The compensatory changes in cardiac output most thoroughly studied are the cardiovascular consequence of normovolemic anemia. When intravascular volume is stable or high after the development of anemia (as opposed to hypovolemic anemia and shock), increases in cardiac output have been consistently reported. Indeed, an inverse relationship between hemoglobin level (or hematocrit) and cardiac output has been clearly established in well-controlled laboratory studies (Fig 9-3).<sup>13,16-19</sup> Similar clinical observations have been made in the perioperative setting<sup>20</sup> and for chronic anemia.<sup>16</sup> Unfortunately, the strength of inferences from clinical studies is limited by confounding factors arising from major coexisting illnesses such as cardiac disease, a lack of appropriate control patients, and significant weaknesses in study design. Researchers have attempted to determine the level of anemia at which cardiac output begins to rise. Reported thresholds for this phenomenon identified in primary clinical and laboratory studies have ranged from 7 to 12 g/dL of hemoglobin.<sup>16</sup> Two major mechanisms are thought to be responsible for the physiologic processes underlying increased cardiac output during normovolemic anemia: 1) reduced blood viscosity and 2) increased sympathetic stimulation of the cardiovascular effectors.<sup>21</sup> Blood viscosity affects both preload and afterload,

two major determinants of cardiac output,<sup>21-23</sup> whereas sympathetic stimulation primarily increases the two other determinants, heart rate and contractility. Unlike the situation for hypovolemic anemia, in this setting the effects of blood viscosity appear to predominate.<sup>22,23</sup>

Complex interactions exist among blood flow, blood viscosity, and cardiac output. In vessels, blood flow alters whole-blood viscosity, and blood viscosity modulates cardiac output. Under experimental conditions in a rigid hollow cylinder, blood flow is directly related to the fourth power of the diameter and to driving pressure. It is inversely related to the length of the vessel and to blood viscosity (Poiseuille-Hagen law).<sup>7,21</sup> Also, blood viscosity increases as flow decreases because of increasing aggregation of red cells. Thus, viscosity is highest in postcapillary venules, where flow is the lowest, and viscosity is lowest in the aorta, where flow is the highest. In postcapillary venules, there is a disproportionate decrease in blood viscosity as anemia worsens, consequently augmenting venous return at a given venous pressure. If cardiac function is normal, the increase in venous return or left ventricular preload is the most important determinant of increased cardiac output during normovolemic anemia. The conclusion is based on experiments in which viscosity was maintained during anemia by means of high-viscosity colloidal solutions. In such studies, the cardiovascular effects of hemodilution were attenuated<sup>22</sup> compared with similar levels of hemodilution accompanied by reduced whole-blood viscosity. Decreased left ventricular afterload, another cardiac consequence of decreased blood viscosity, also may be an important mechanism for the increase in cardiac output as anemia worsens.<sup>22</sup>

Sympathetic stimulation can result in increased cardiac output through enhanced myocardial contractility<sup>24</sup> and increased venomotor tone.<sup>18,25</sup> The effects of anemia on left ventricular contractility in isolation have not been clearly determined, given the complex changes in preload, afterload, and heart rate. Only one before-and-after hemodilution study was performed with load-independent measures to document increased left ventricular contractility.<sup>24</sup> Chapler and Cain<sup>18</sup> summarized several well-controlled animal studies indicating that venomotor tone increases as a result of stimulation of the aortic chemoreceptors. If sympathetic stimulation is significant in the specific clinical setting, contractility is increased from stimulation of the  $\beta$ -adrenergic receptors.<sup>21,26</sup>

The inverse relationship between cardiac output and hemoglobin level has led investigators to attempt to determine the hemoglobin level that maximizes oxygen transport. Richardson and Guyton<sup>27</sup> evaluated the effects of hematocrit on cardiac performance in a canine model. They established that optimal oxygen transport occurred between hematocrits of 40% and 60%. Others determined maximum oxygen delivery to be in the lower end of the range, at a hematocrit of 40% to 45% (hemoglobin 13 to 15 g/dL).<sup>28,29</sup> However, in one of the most widely quoted studies addressing this topic,<sup>30</sup> investigators found peak oxygen transport to occur at a hematocrit of 30% (hemoglobin concentration, 10 g/dL). Unfortunately, global indices of optimal oxygen delivery mask any differences in blood flow between

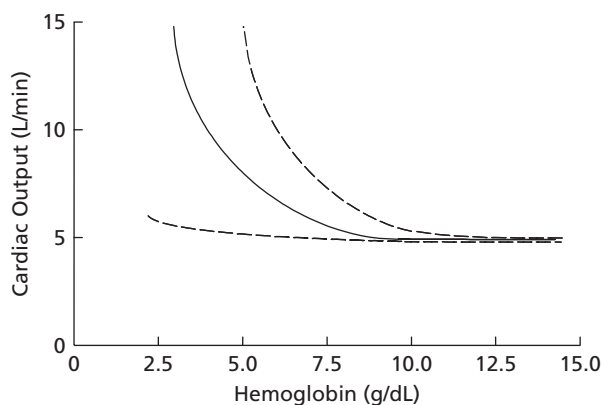


**Figure 9-3.** The theoretic effect of hemoglobin concentration on cardiac output. The curves illustrate how cardiac output increases as hemoglobin concentration decreases. The solid curve is meant to describe the increase in a healthy adult. The dashed line on the top shows how the cardiac output response can be accentuated in a young athlete and the lower dashed line might correspond to someone with poor cardiovascular function.

specific organs.<sup>31,32</sup> In addition, attempting to identify a single optimal hemoglobin concentration that maximizes oxygen delivery neglects the large number of factors interfering with adaptive mechanisms during management of patients other than healthy, young patients with anemia.

Will the transfusion of allogeneic RBCs reverse any adaptive response to acute or chronic normovolemic anemia? Assuming that oxygen-carrying capacity is not impaired during storage and that hematocrit is restored after a transfusion, the cardiovascular consequences will be reversed. However, the storage process alters the properties of red cells. These alterations may impair flow and oxygen release from hemoglobin<sup>9</sup> in the microcirculation.

### Microcirculatory Effects of Anemia and Red Cell Transfusion

At the level of the microcirculation, three putative adaptive mechanisms increase the amount of oxygen supplied to tissues by capillary networks. In a model of the microcirculation proposed by Krogh,<sup>33</sup> oxygen supply to the tissues is enhanced through recruitment of previously closed capillaries, increased capillary flow, and increased oxygen extraction from existing capillaries. The degree of anemia, the specific tissue bed, and a variety of disease processes affect microcirculatory blood flow and oxygen supply.<sup>7,34</sup> As the degree of hemodilution becomes more pronounced and hematocrit decreases, blood viscosity decreases disproportionately in capillary networks. This occurs because the hematocrit is highest in the capillary network, as a consequence, there is a larger decrease in capillary viscosity.

Stored red cells have properties that differ from those of their in-vivo counterparts; many are related to the duration of storage. Characteristically, older RBC units have lower levels of 2,3-DPG. The result is a leftward shift in the oxyhemoglobin dissociation curve, which can impede delivery of oxygen to the tissues.<sup>13</sup> In addition, storage of red cells decreases the deformability of their membrane.<sup>35</sup> As a consequence, stored red cells may impede flow in the microcirculation<sup>36</sup> and may have limited ability to release oxygen to tissues. However, these storage lesions are reversible within 24 to 48 hours.<sup>37</sup>

There are reports<sup>38</sup> suggesting that disease processes such as sepsis impair red cell deformability. In conjunction with significant systemic microcirculatory dysfunction, the decrease in red cell deformability may dramatically affect tissue oxygen delivery in sepsis and septic shock.<sup>38</sup> This body of evidence suggests that RBC transfusions increase systemic oxygen delivery but may have adverse effects on microcirculatory flow.

### Interaction between Pathophysiologic Processes and Anemia

Several disease processes affecting either the entire body or specific organs potentially limit adaptive responses and make patients more vulnerable to the effects of anemia. Specifically, heart, lung, and cerebrovascular diseases have been proposed to increase the risk of adverse consequences of anemia. Age, severity

of illness, and therapeutic interventions also may affect adaptive mechanisms.

The heart, specifically the left ventricle, is particularly prone to adverse consequences of anemia. This is because the myocardium consumes 60% to 75% (extraction ratio) of all oxygen delivered by the coronary circulation.<sup>28,31,39</sup> Such a high extraction ratio is unique to the coronary circulation. As a result, oxygen delivery to the myocardium can increase substantially only with an increase in blood flow.<sup>40</sup> In addition, most left ventricular perfusion is restricted to the diastolic period, because pressures inside the left ventricle are too high to allow adequate coronary blood flow during systole. Thus, any shortening of its duration (eg, tachycardia) decreases blood flow. Laboratory studies have been performed to investigate the effects of normovolemic anemia on the coronary circulation.<sup>29,39,41</sup> There appear to be minimal consequences of anemia with hemoglobin levels in the range of 7 g/dL if the coronary circulation is normal.<sup>19,24</sup> However, myocardial dysfunction and ischemia either occur earlier or are greater in anemic animal models with moderate to high-grade coronary stenosis compared with controls with normal hemoglobin values.<sup>42</sup>

Data from studies with human subjects are inconsistent. Several clinical studies involving patients with coronary artery disease undergoing normovolemic hemodilution have not shown any increase in cardiac complications or silent ischemia during electrocardiographic monitoring.<sup>43</sup> In addition, a retrospective analysis involving 224 patients undergoing CABG surgery did not show a significant association between hemoglobin level and coronary sinus lactate level (an indicator of myocardial ischemia).<sup>44</sup> However, in two recent cohort studies, moderate anemia was poorly tolerated by perioperative<sup>45</sup> and critically ill patients<sup>46</sup> with cardiovascular disease. Thus, retrospective studies seem to support preclinical reports. It is also plausible that anemia results in considerable increases in morbidity and mortality among patients with other cardiac diseases, including heart failure and valvular heart disease, presumably because of the greater burden of the adaptive increase in cardiac output.

During normovolemic anemia, cerebral blood flow increases as hemoglobin values decrease. Investigators have observed increases ranging from 50% to 500% of baseline value in laboratory studies<sup>47</sup> and in one study with human subjects.<sup>48</sup> Cerebral blood flow increases because of overall increases in cardiac output, which is preferentially diverted to the cerebral circulation. As oxygen delivery begins to decrease, cerebral tissues are able to increase the amount of oxygen extracted from blood. A number of factors, including degree of hemodilution, type of fluid used for volume expansion, volume status (preload), and extent of cerebrovascular disease, are capable of potentially modifying global or regional cerebral blood flow during anemia.<sup>49</sup> The increase in global cerebral blood flow combined with the potential for improved flow characteristics across areas of vascular stenosis (improved rheologic properties of blood because of decreased viscosity) prompted a number of laboratory and clinical<sup>49-52</sup> studies to investigate hemodilution as therapy for acute ischemic stroke.<sup>50-52</sup>



The results of laboratory studies suggest that moderate degrees of anemia alone should rarely result in or worsen cerebral ischemia. None of the randomized clinical trials demonstrated that hemodilution in acute ischemic stroke improved clinical outcomes in patients. Because of the variety of variables that affect clinical outcomes, the negative findings may not fully rule out the possibility that hemodilution offers therapeutic benefit. Thus, the currently available evidence indicates that cerebrovascular disease does not appear to predispose patients to serious morbidity from anemia.

Changes in oxygen delivery to the brain during normovolemic anemia (either increases or decreases in blood flow) do not uniformly affect various cerebral pathologic conditions. For example, patients with high intracranial pressure from traumatic brain injury may be adversely affected by increased cerebral blood flow. However, after subarachnoid hemorrhage, mild degrees of normovolemic or hypervolemic anemia may improve overall oxygen delivery, possibly by overcoming the effects of cerebral vasospasm and thereby improving cerebral blood flow through decreased viscosity.<sup>53</sup> However, the effects of moderate to severe anemia in subarachnoid hemorrhage have not been assessed in laboratory or clinical studies.

One of the major consequences of redistributing some of the available cardiac output toward the coronary and cerebral circulation during normovolemic anemia is the shunting of flow away from other organs, including the kidneys and intestines. Critically ill patients may be adversely affected by this redistribution,<sup>54</sup> which could result in increased intestinal ischemia, bacterial translocation, and multiple-system organ failure.<sup>55</sup> Critical illness also can tax many of the body's adaptive responses. Specifically, cardiac performance may be impaired<sup>56</sup> or may already be at maximal capacity in response to increased metabolic demands. Pathologic processes affecting the microcirculation, particularly prevalent among critically ill patients, also may affect the patient's response to anemia and transfusions.

## Clinical Outcomes of Anemia and Red Cell Transfusion

Every medical decision must weigh risk vs benefit. The decision to administer RBC units must consider the risks of blood transfusion (see Chapters 44 through 56), the risk of anemia, and the level of anemia at which blood transfusion prevents the associated adverse outcomes.

### Risk of Anemia

#### Preclinical Laboratory Studies

The critical hemoglobin threshold is similar in different animals.<sup>1</sup> Results of studies suggest that healthy animals can tolerate hemoglobin levels between 3 and 5 g/dL after normovolemic hemodilution. Electrocardiographic changes consistent with ischemia occur at hemoglobin levels less than 5 g/dL, whereas

lactate production, depressed ventricular function, and death have occurred at hemoglobin levels of 3 g/dL or less. Some animals survive with hemoglobin levels as low as 1 to 2 g/dL.<sup>57</sup> However, results of studies with animals suggest a decreased ability to tolerate anemia in the presence of cardiac disease. In dogs with experimentally induced coronary stenosis varying from 50% to 80%, ST-segment changes or locally depressed cardiac function occurred at hemoglobin levels in the range of 7 to 10 g/dL.<sup>58,59</sup>

#### Human Studies

Studies involving patients who refuse blood transfusion for religious reasons provide critical insight into the effect of anemia on humans. The largest study was performed with 1958 adult surgical patients who refused transfusion for religious reasons.<sup>45</sup> The mortality was greatest among patients with the lowest preoperative hemoglobin concentrations. Among patients with underlying cardiovascular disease, the risk of death was markedly greater than for patients without cardiovascular disease, especially in those patients with a hemoglobin concentration less than 10 g/dL. Among patients without underlying cardiovascular disease, the difference in mortality at hemoglobin levels greater than or less than 10 g/dL was not as great (Fig 9-4). None of the 99 patients with hemoglobin concentrations between 7 and 8 g/dL died.<sup>60</sup> However, the risk of death rose sharply below a hemoglobin concentration of 5 g/dL. These results, as well as data on animals and physiologic data, suggest that anemia is not tolerated as well in the presence of cardiovascular disease.

In a series of studies, the effect of anemia was evaluated among healthy volunteers who underwent isovolemic reduction of hemoglobin level to 5 g/dL. Transient and asymptomatic electrocardiographic changes were found in five of the 87 volunteers

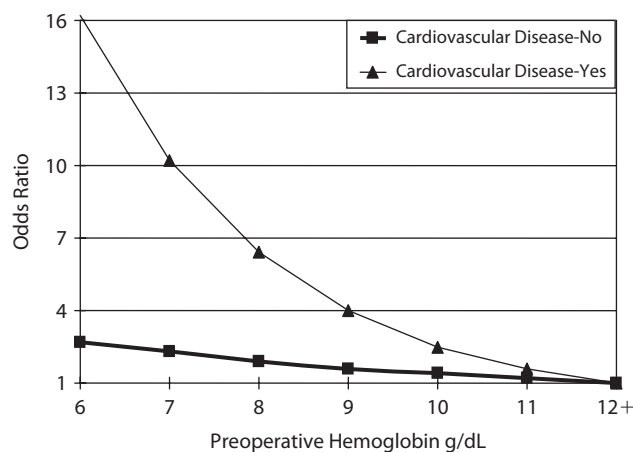


**Figure 9-4.** Association between preoperative hemoglobin level and mortality among patients with and without cardiovascular disease.<sup>45</sup> In a population of patients who refused blood transfusion, the risk of death was higher among patients with cardiovascular disease (top line) than among patients without cardiovascular disease (bottom line) for each preoperative hemoglobin level. (Used with permission from Carson et al.<sup>45</sup>)

included in two studies.<sup>61,62</sup> These changes occurred when the hemoglobin level was between 5 and 7 g/dL and in patients with faster heart rates.<sup>62</sup> Changes in critical oxygen delivery were not measured. Subtle but reversible changes in cognition were identified in nine volunteers younger than 35 years at a hemoglobin level between 5 and 7 g/dL.<sup>63</sup> Self-rated fatigue was found in eight volunteers when the hemoglobin level decreased to 7 g/dL. Fatigue increased as hemoglobin levels decreased to 5 g/dL.<sup>64</sup> The results of these studies suggest that important clinical effects can be detected in young, healthy humans with hemoglobin levels between 5 and 7 g/dL.

It is uncertain how these results apply to older patients with comorbid factors who are also under stress from surgery or acute illness. An analysis of 31,000 patients 65 years of age or older undergoing major noncardiac surgery examined the association between preoperative hematocrit and mortality or cardiac morbidity (cardiac arrest or Q-wave myocardial infarction).<sup>65</sup> Mortality rose monotonically when the hematocrit was less than 36%. Cardiac events were more frequent when the hematocrit was less than 39%. These results, in contrast to experimental data, suggest that mild anemia is associated with increased mortality and morbidity. However, anemia may be only a marker of underlying disease and studies are needed to demonstrate improved outcome if anemia is corrected by transfusion.

## Efficacy of Transfusion

### Observational Studies

Selected observational studies of the effect of RBC transfusion on clinical outcomes are described in Table 9-2. The results vary, although only one study has demonstrated a beneficial effect of RBC transfusion. The validity of these studies is uncertain because the decision to transfuse often correlates with the illness burden of the patient. It is likely that comorbidity was not adequately adjusted for in these studies. For example, there are many observational studies demonstrating that blood transfusion increases risk of bacterial infection,<sup>76</sup> yet none of the randomized clinical trials have confirmed this finding. Only randomized clinical trials can overcome this limitation.

### Clinical Trials in Adults

Ten randomized clinical trials contrasted the effects of different transfusion thresholds (Table 9-3).<sup>77-86</sup> The clinical settings varied, although each trial randomly assigned patients to transfusion on the basis of a restrictive or a liberal strategy. Restrictive triggers (specified hemoglobin concentrations that had to be attained) ranged from 7 to 9 g/dL.

Two trials specified a hematocrit of 25% or 30% for RBC transfusion. The liberal transfusion strategies specified the following triggers: 100% of normal red cell volume, two RBC units (immediately in one trial, postoperatively in another) irrespective of clinical state, and transfusion sufficient to maintain hemoglobin level at or above 10 g/dL in three trials and above 9 g/dL in another. Two trials specified the liberal triggers as transfusion to maintain hematocrit at 32% and 40% or greater. There is

overlap between the liberal and restrictive transfusion groups in these trials. One trial involving patients in the intensive care unit (ICU) contributed 47% of the patients and 82% of the recorded deaths. There were a total of 1780 trial participants.

Of the 10 trials, only three included more than 100 patients, and only one of these evaluated a transfusion strategy that included assessment for symptoms. In one of these trials, 428 patients undergoing first-time, elective CABG surgery were randomly assigned to study arms with transfusion triggers of 9 g/dL vs 8 g/dL.<sup>85</sup> The differences between perioperative hemoglobin levels were small, and the mean reduction in hemoglobin level during the admission was equal between the groups. The event rates were very low, and there were no differences in any outcome. The second trial included 127 patients undergoing knee arthroplasty. Patients were randomly assigned to receive autologous blood transfusion immediately after the operation (first unit in recovery room and second unit on return to the ward) vs autologous blood only if the hemoglobin level decreased to less than 9 g/dL.<sup>86</sup> The mean postoperative hemoglobin level was approximately 0.7 g/dL different, although only 25% of the restrictive group received a transfusion. There were no differences in outcome. In a third trial (pilot study), 84 patients with hip fracture undergoing surgical repair were randomly assigned to groups receiving either transfusion at a 10 g/dL threshold or transfusion for symptoms (transfusion was also allowed if hemoglobin level was less than 8 g/dL).<sup>83</sup> The mean hemoglobin level before randomization was 9.1 g/dL. The lowest mean hemoglobin level after randomization in the group with symptoms was 8.8 g/dL, and the highest mean hemoglobin level in the threshold group was 11.1 g/dL. There were no differences in outcomes (including functional recovery, mortality, and morbidity), although 60 days after the operation, five patients in the symptomatic group had died, as had two patients in the 10 g/dL group. In all of these trials (and the other trials in Table 9-3), the numbers of patients were much too small to evaluate the effect of lower transfusion triggers on clinically important outcomes such as mortality, morbidity, and functional recovery.

The Transfusion Requirements in Critical Care (TRICC) trial<sup>81,84</sup> is the only adequately powered study to evaluate clinically important outcome. In the main study, the investigators randomly assigned 838 volume-resuscitated ICU patients to a restrictive strategy in which patients received allogeneic RBC transfusions at hemoglobin levels of 7 g/dL (and were maintained between 7 to 9 g/dL) or to a liberal strategy of receiving RBCs at 10 g/dL (and were maintained between 10 and 12 g/dL). Average hemoglobin levels (8.5 vs 10.7 g/dL) and RBC units transfused (2.6 vs 5.6 units) were significantly lower in the restrictive as opposed to the liberal group. The 30-day mortality was slightly lower in the restrictive transfusion group (18.7% vs 23.3%), although the finding was not statistically significant ( $p = 0.11$ ). Mortality was lower in the restrictive transfusion group in patients less than 55 years of age ( $p < 0.02$ ) and less ill patients defined by APACHE II scores less than 20 ( $p < 0.02$ ). Furthermore, the restrictive transfusion group had fewer patients

**Table 9-2.** Summary of Nonrandomized Studies

| Study                               | Clinical Setting                   | Subjects                                                                                                                                                                                                                                                                               | Outcomes                                                                                                                                                                                                                                                                         |
|-------------------------------------|------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Nelson et al (1993) <sup>66</sup>   | Vascular surgery (n=27)            | High-risk patients who had undergone elective infra-inguinal bypass vascular procedures<br>Anemic group: n=13<br>Nonanemic group: n=14                                                                                                                                                 | MI occurred in 14% of pts with Hct >28% compared to 77% with Hct <28%<br>No deaths in either group                                                                                                                                                                               |
| Hébert et al (1997) <sup>67</sup>   | Critical care (n=4,470)            | Critically ill patients admitted to ICU<br>Survivors: n=3,469<br>Nonsurvivors: n=1,001                                                                                                                                                                                                 | Survivors had higher hemoglobin levels than nonsurvivors                                                                                                                                                                                                                         |
| Paone et al (1997) <sup>68</sup>    | Cardiac surgery (n=100)            | Patients undergoing isolated CABG surgery<br>Transfusion group: n=13<br>No transfusion group: n=87<br><br>Patients received allogeneic RBCs on bypass for low SvO <sub>2</sub> (<55%) and transfused postoperatively for a Hct <20% or at any Hct level if deemed clinically warranted | No difference in clinical outcomes<br>Length of ICU stay longer in transfused patients (2.6±0.3 days) vs nontransfused group (1.6±0.1 days)                                                                                                                                      |
| Hogue et al (1998) <sup>69</sup>    | Urologic surgery (n=190)           | Patients undergoing radical prostatectomy<br>Hct <28% group: n=81<br>Hct ≥28% group: n=100                                                                                                                                                                                             | Patients with Hct levels <28% immediately after surgery were significantly (p = 0.05) more likely to have ischemic episodes                                                                                                                                                      |
| Carson et al (1998) <sup>70</sup>   | Orthopedic surgery (n=8,787)       | Hip fracture patients, aged 60 years or older, who underwent surgical repair<br>Transfused group: n=3,699<br>Nontransfused group: n=5,088                                                                                                                                              | No difference in 30- or 90- day mortality                                                                                                                                                                                                                                        |
| Spieß et al (1998) <sup>71</sup>    | Cardiac surgery (n=2,202)          | CABG surgery patients, mean age ±SD = 64.3 ± 10.2 years<br>High ICU Hct ≥34% group: n=410<br>Medium ICU Hct group 25% to 33%: n=1,544<br>Low ICU Hct ≤24% group: n=248                                                                                                                 | MI highest in ICU Hct (8.3%) then medium ICU Hct (5.5%) and low ICU Hct (3.6%)                                                                                                                                                                                                   |
| Wu et al (2001) <sup>72</sup>       | Myocardial infarction (n=78,794)   | MI patients 65 years old or older<br>5.0 to 24.0% n=380<br>24.1 to 27.0% n=838<br>27.1 to 30.0% n=2,106<br>30.1 to 33.0% n=4,848<br>33.1 to 36.0% n=9,885<br>36.1 to 39.0% n=16,218<br>39.1 to 48.0% n=44,699                                                                          | Transfusion was associated with reduction in 30-day mortality in patients with Hct 5.0% to 33%                                                                                                                                                                                   |
| Vincent et al (2002) <sup>73</sup>  | Critical care (n=1,136)            | 41% of patients transfused during 28-day period                                                                                                                                                                                                                                        | Transfusion was associated with increased mortality; odds ratio 1.37; 95% confidence interval 0.02-1.84                                                                                                                                                                          |
| Rao et al (2004) <sup>74</sup>      | Acute coronary syndrome (n=24,112) | Subjects included in three clinical trials<br>Received transfusion: n=2,400<br>No transfusion: n=21,711                                                                                                                                                                                | Transfusion was associated with an increased 30-day mortality (hazard ratio, 3.94; 95% confidence interval, 3.26-4.75) and 30-day mortality or MI (hazard ratio, 2.92; 95% confidence interval, 2.55-3.35)                                                                       |
| Sabatine et al (2005) <sup>75</sup> | Acute coronary syndrome (n=39,922) | Subjects from 16 clinical trials<br>4.6% of patients with ST-segment elevated MI received transfusion<br><br>2.7% of patients with non-ST-segment elevated MI received transfusion                                                                                                     | In ST-segment elevated MI, transfusion was associated with a decreased risk of cardiovascular death when hemoglobin <12 g/dL<br><br>In non-ST-segment elevated MI, transfusion was associated with increased risk of cardiovascular death regardless of hemoglobin concentration |

Hct = hematocrit; ICU Hct = intensive care unit hematocrit; MI = myocardial infarction; CABG = coronary artery bypass graft.

**Table 9-3.** Results of Randomized Controlled Trials in Adults

| Study                              | Setting                          | Subjects: Eligibility and Comparability                                                                                                                                          | Transfusion Strategy                                                                                                                                                                                                                                                   | Mean Blood Usage, Units/Pt (sd)                                                                   | Proportion Transfused (n) | Mean Hb / Hct Levels (sd)                                                                                                 | 30-Day Mortality (n) | Length of Hospital Stay (mean $\pm$ sd)                            |
|------------------------------------|----------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|---------------------------|---------------------------------------------------------------------------------------------------------------------------|----------------------|--------------------------------------------------------------------|
| Topley et al (1956) <sup>77</sup>  | Trauma (n=22)                    | > 1 L blood loss; considered to be at no clinical risk in raising the blood volume $\geq$ 100% of normal, or allowing it to reach 30% below normal                               | <u>Liberal</u> : to achieve red cell volume $\geq$ 100% of normal<br><u>Restrictive</u> : maintain red cell volume 70%–80% of normal                                                                                                                                   | 11.3 (6.9)<br>4.8 (6.7)                                                                           | 100% (10)<br>67% (8)      | Lowest Hb: 15.6 $\pm$ 2.0 g/dL<br>Lowest Hb: 11.3 $\pm$ 0.7 g/dL                                                          | –<br>–               | –<br>–                                                             |
| Blair et al (1986) <sup>78</sup>   | GI bleeding (n=50)               | Acute severe upper gastrointestinal hemorrhage                                                                                                                                   | <u>Liberal</u> : patients received at least 2 RBC units immediately on admission to hospital<br><u>Restrictive</u> : patients did not receive RBCs during the first 24 hours unless Hb <8.0g/dL or shock persisted after initial resuscitation with colloid            | 4.6 (1.5)<br>2.6 (3.1)                                                                            | 100% (24)<br>19.2% (5)    | Admission Hct: 28 (5.9)%<br>Discharge Hct: 37.0 (7.8)%<br>Admission Hct: 29 (8.2)%<br>Discharge Hct: 37.0 (7.1)%          | 8.3% (2)<br>0% (0)   | –<br>–                                                             |
| Fortune et al (1987) <sup>79</sup> | Trauma / acute hemorrhage (n=25) | Patients who had sustained a Class III or Class IV hemorrhage and had clinical signs of shock                                                                                    | <u>Liberal</u> : Hct was brought up to 40% slowly over a period of several hours by the infusion of RBCs<br><u>Restrictive</u> : Hct was kept close to 30% by the administration of RBCs                                                                               | –<br>–                                                                                            | –<br>–                    | Average Hct for 3-day period: 38.4 (2.1)%<br>Average Hct for 3-day period: 29.7 (1.9)%                                    | –<br>–               | –<br>–                                                             |
| Johnson et al (1992) <sup>80</sup> | Cardiac surgery (n=38)           | Patients undergoing elective coronary revascularization and able to doante at least 3 RBC units preoperatively                                                                   | <u>Liberal</u> : patients received blood transfusion to achieve a Hct value of 32% so long as autologous blood was available<br><u>Restrictive</u> : patients received transfusion only if the Hct value fell below 25%                                                | 2.05 (0.93)<br>1.0 (0.86)                                                                         | 100% (18)<br>75% (15)     | Hct at 4 hours after surgery: 31.3%<br>Hct at 4 hours after surgery: 28.7%                                                | –<br>–               | 7.6 (1.9) days<br>7.9 (4.3) days                                   |
| Hébert et al (1995) <sup>81</sup>  | Critical care (n=69)             | Critically ill patients admitted to one of five tertiary level ICUs with normovolemia concentrations after initial treatment who had Hb concentrations <9.0 g/dL within 72 hours | <u>Liberal</u> : patients received RBCs if their Hb was $\leq$ 10.0 to 10.5 g/dL; Hb concentration maintained at 10.0 to 12.0 g/dL<br><u>Restrictive</u> : patients received RBCs only if their Hb was 7.0 to 7.5 g/dL; Hb concentration maintained at 7.0 to 9.0 g/dL | Mean units per patient: 4.8<br>Total units: 174<br>Mean units per patient: 2.5<br>Total units: 82 | –<br>–                    | Admission Hb: 9.3 (1.3) g/dL<br>Average daily Hb: 10.9 g/dL<br>Admission Hb: 9.7 (1.4) g/dL<br>Average daily Hb: 9.0 g/dL | 25% (9)<br>24% (8)   | Median (IQR)<br>31 (13–64) days<br>Median (IQR)<br>38 (25–62) days |
| Bush et al (1997) <sup>82</sup>    | Vascular surgery (n=99)          | Patients undergoing elective aortic and infra-inguinal arterial reconstruction                                                                                                   | <u>Liberal</u> : patients received RBCs to maintain Hb > 10.0 g/dL<br><u>Restrictive</u> : Patients received RBCs only if Hb level fell below 9.0 g/dL                                                                                                                 | Total units: 3.7 (3.5)<br>Intraop: 2.4 (2.5)<br>Total units: 2.8 (3.1)<br>Intraop: 1.5 (1.7)      | 88% (43)<br>80% (40)      | Hb during 48-hour postop. period: 11.0 (1.2) g/dL<br>Hb during 48-hour postop. period: 9.8 (1.3) g/dL                     | 8% (4)<br>8% (4)     | 11 (9) days<br>10 (6) days                                         |
| Carson et al (1998) <sup>83</sup>  | Orthopedic surgery (n=84)        | Hip fracture patients undergoing surgical repair who had postoperative Hb levels < 10.0 g/dL                                                                                     | <u>Liberal</u> : patients received 1 RBC unit at the time of random assignment and then as needed to maintain Hb > 10.0 g/dL                                                                                                                                           | Total median: 2 (1–2)                                                                             | 98.8% (83)                | Lowest Hb: 9.4 (1.0) g/dL                                                                                                 | 2.4% (1)             | 6.3 (3.4) days                                                     |

(Continued)

Table 9-3. (Continued)

| Study                             | Setting                    | Subjects: Eligibility and Comparability                                                                                                                                             | Transfusion Strategy                                                                                                                                                                                                                                                                                                                 | Mean Blood Usage, Units/Pt (sd)       | Proportion Transfused (n)             | Mean Hb / Hct Levels (sd)                                            | 30-Day Mortality (n) | Length of Hospital Stay (mean $\pm$ sd) |
|-----------------------------------|----------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------|---------------------------------------|----------------------------------------------------------------------|----------------------|-----------------------------------------|
| Hébert et al (1999) <sup>84</sup> | Critical care (n=838)      | Critically ill patients admitted to one of 22 tertiary level and three community ICUs with normovolemia after initial treatment who had Hb concentrations <9.0 g/dL within 72 hours | Restrictive: transfusion was delayed until the patient developed symptoms or consequences of anemia, or Hb value <8.0 g/dL in the absence of symptoms                                                                                                                                                                                | Total median: 0 (0-2)                 | 45.2% (38)                            | Lowest Hb: 8.8 (1.2) g/dL                                            | 2.4% (1)             | 6.4 (3.4) days                          |
|                                   |                            |                                                                                                                                                                                     | Liberal: patients received RBCs to maintain Hb concentration at 10.0 to 12.0 g/dL                                                                                                                                                                                                                                                    | Total: 5.6 (5.3)                      | 100% (420)                            | Hb: (mean daily) 10.7 (0.7) g/dL                                     | 23.3% (98)           | 35.5 (19.4) days                        |
|                                   |                            |                                                                                                                                                                                     | Restrictive: patients received RBCs to maintain Hb concentration at 7.0 to 9.0 g/dL.                                                                                                                                                                                                                                                 | Total: 2.6 (4.1)                      | 77% (280)                             | Hb: (mean daily) 8.5 (0.7) g/dL                                      | 18.7% (78)           | 34.8 (19.5) days                        |
| Brace et al (1999) <sup>85</sup>  | Cardiac surgery (n=428)    | Patients undergoing first-time elective coronary revascularisation                                                                                                                  | Liberal: patients received RBC transfusions on the instructions of their individual physicians, who considered the clinical assessment of the patient and the institutional guidelines, which propose a Hb level < 9.0 g/dL as the postoperative threshold for RBC transfusion                                                       | Postop: 1.4 (1.8)<br>Total: 2.5 (2.6) | Postop: 48% (104)<br>Total: 64% (138) | Hb: mean net reduction in Hb (admission to discharge) 4.2 (1.9) g/dL | 2.7% (6)             | 7.9 (4.9) days                          |
|                                   |                            |                                                                                                                                                                                     | Restrictive: received an RBC transfusion in the postoperative period for a Hb level < 8.0 g/dL, unless the patient experienced                                                                                                                                                                                                       | Postop: 0.9 (1.5)<br>Total: 2.0 (2.2) | Postop: 35% (74)<br>Total: 60% (127)  | Hb: mean net reduction in Hb (admission to discharge) 4.2 (1.7) g/dL | 1.4% (3)             | 7.5 (2.9) days                          |
|                                   |                            |                                                                                                                                                                                     | <ul style="list-style-type: none"> <li>• blood loss &gt; 750 mL since the last transfusion</li> <li>• hypovolemia with hemodynamic instability and excessive acute blood loss</li> <li>• acute respiratory failure or inadequate cardiac output and oxygenation</li> <li>• hemodynamic instability requiring vasopressors</li> </ul> |                                       |                                       |                                                                      |                      |                                         |
| Lotke et al (1999) <sup>86</sup>  | Orthopedic surgery (n=127) | Patients undergoing primary total knee arthroplasty who were able to donate 2 units of autologous blood preoperatively                                                              | Liberal: patients received their autologous blood immediately after surgery, the 1st unit beginning in the recovery room and the 2nd unit delivered on return to the ward                                                                                                                                                            | –                                     | Postop: 100% (65)                     | Mean postop. Hb: Day 1 (11.4) g/dL<br>Day 3 (10.7) g/dL              | –                    | –                                       |
|                                   |                            |                                                                                                                                                                                     | Restrictive: patients received all their autologous blood if their Hb level had fallen below 9.0 g/dL                                                                                                                                                                                                                                | –                                     | Postop: 26% (16)                      | Mean postop. Hb: Day 1 (10.6) g/dL<br>Day 3 (10.0) g/dL              | –                    | –                                       |
|                                   |                            |                                                                                                                                                                                     |                                                                                                                                                                                                                                                                                                                                      |                                       |                                       |                                                                      |                      |                                         |

Hb = hemoglobin; Hct = hematocrit; GI = gastrointestinal; RBCs = Red Blood Cells; ICU = intensive care unit; IQR = interquartile range.

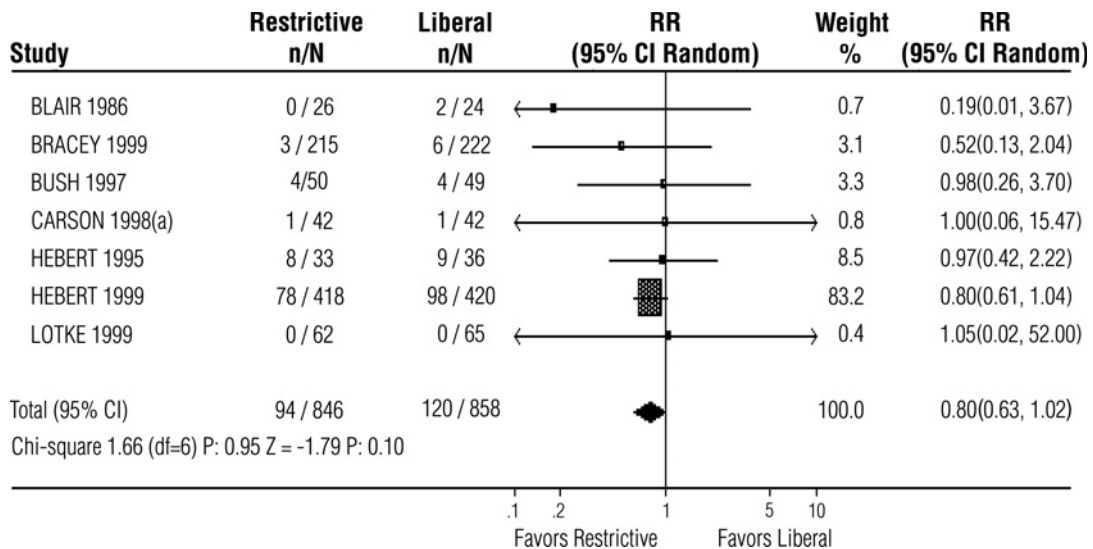


**Figure 9-5.** Effect of restrictive transfusion triggers on 30-day all-cause mortality. Summary of data from randomized clinical trials comparing effect of restrictive vs liberal transfusion strategies on mortality (expressed as relative risks). (Used with permission from Carson et al.<sup>87</sup>)

with myocardial infarction (0.07% vs 2.9%;  $p = 0.02$ ) and congestive heart failure (5.3% vs 10.7%;  $p < 0.01$ ).

A meta-analysis combined data from five or more trials for six outcomes: probability of RBC unit transfusion, volume of red cells transfused, hematocrit, cardiac events, mortality at 30 days, and overall length of hospital stay.<sup>87</sup> The pooled data indicated that on average, a restrictive transfusion trigger reduced the probability of RBC transfusion by a proportional 42% (an average saving of 0.93 RBC unit per transfused patient) and resulted in a hematocrit 5.6% lower on average than in patients who received more liberal transfusions. The effects on length of hospital stay and the rate of cardiac events were not increased significantly by the use of restrictive transfusion triggers. Restrictive transfusion triggers were not associated with an increase in mortality (Fig 9-5). The TRICC trial<sup>84</sup> involving ICU patients contributed 83% of the information on mortality data in the meta-analysis.

Functional recovery is another potentially important benefit of higher hematocrit. Only one small pilot study evaluated the effect of RBC unit transfusion on the functional ability of patients with anemia.<sup>73</sup> The number of subjects was too small to detect clinically important differences. The results from the FOCUS trial, which will evaluate functional recovery in up to 2000 hip fracture patients with cardiovascular disease or cardiovascular risk factors, should be available in 2009.<sup>88</sup> Most of the other published data relating anemia to function were generated from clinical trials performed to evaluate the use of recombinant human erythropoietin in end-stage renal failure and patients undergoing cancer chemotherapy. These limited data suggest that increasing the hemoglobin level of patients with marked anemia (hemoglobin,  $<10$  g/dL) increases exercise tolerance.<sup>89</sup> The studies with negative findings<sup>90</sup> evaluated the effect of increasing hemoglobin level beyond the 10 g/dL threshold. A

review of the use of erythropoietin in the care of patients with cancer-related anemia<sup>90</sup> concluded that patients receiving erythropoietin had increased levels of energy and function. Two recent clinical trials compared use of erythropoietin to achieve a high hemoglobin concentration (13-15 g/dL) vs a lower hemoglobin concentration (10.5-11.5 g/dL) in patients with chronic renal insufficiency.<sup>91,92</sup> Only one study found improvement in function and quality of life measures.<sup>92</sup> The United States Food and Drug Administration has concluded that there is insufficient evidence to conclude that erythropoietin-stimulating agents alleviate fatigue or increase energy.

### Clinical Trials in Children

Blood is frequently transfused in critically ill infants and children. In a recent survey, 14% of patients in pediatric ICUs received blood transfusion.<sup>93</sup> There have been three clinical trials evaluating liberal vs restrictive transfusion thresholds in this population (see Table 9-4). One hundred hospitalized preterm infants with birthweights between 500 to 1300 g were randomly assigned to two transfusion levels.<sup>94</sup> The transfusion protocol adjusted the hematocrit level that led to transfusion depending on the respiratory status of the infant. A primary outcome was not designated among the 15 clinical events evaluated. Infants in the restrictive group received a median of 2 units less than the liberal group during the study and the mean difference in hemoglobin concentration was  $\sim 2$  g/dL. There were no differences between the liberal and restrictive transfusion groups for most outcomes including survival, patent ductus arteriosus, retinopathy, or bronchopulmonary dysplasia. Infants assigned to the restrictive group had more apneic events and more neurologic events (combined parenchymal brain hemorrhage or periventricular leukomalacia). These differences in outcomes should be interpreted as

**Table 9-4.** Results of Randomized Controlled Trials in Children

| Study                                | Setting                               | Subjects: Eligibility and Comparability                                                               | Transfusion Strategy                                                                 | Mean Blood Usage, Units/Pt (sd) | Proportion Transfused (n) | Mean Hb / Hct Levels (sd)                                                                                                              | Primary Outcome %                                                      | 30-Day Mortality (n)           | Other Outcomes % (n)                                                                                                                                                                                                                   | Length of Hospital Stay ( $\pm$ sd)                |
|--------------------------------------|---------------------------------------|-------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|---------------------------------|---------------------------|----------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------|--------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------|
| Bell et al (2005) <sup>94</sup>      |                                       | 100 preterm infants with birthweights of 500-1300 g                                                   | <u>Liberal</u> : Algorithm of high transfusion thresholds                            | 5.2 (4.5)                       | 88%                       | Not reported                                                                                                                           | No outcome designated as primary outcome among the 15 events evaluated | 98%                            | <ul style="list-style-type: none"> <li>Patent ductus arteriosis, 39%</li> <li>Intraventricular hemorrhage, 33%</li> <li>Periventricular leukomalacia, 0%</li> <li>Retinopathy 60%</li> <li>Bronchopulmonary dysplasia, 38%</li> </ul>  | Median: 74 (54-96) days                            |
|                                      |                                       |                                                                                                       | <u>Restrictive</u> : Algorithm of low transfusion thresholds                         | 3.3 (2.9)                       | 90%                       | Not reported                                                                                                                           |                                                                        | 96%                            | <ul style="list-style-type: none"> <li>Patent ductus arteriosis, 31%</li> <li>Intraventricular hemorrhage, 29%</li> <li>Periventricular leukomalacia, 14%</li> <li>Retinopathy, 51%</li> <li>Bronchopulmonary dysplasia 35%</li> </ul> | Median: 73 (62-95) days                            |
| Kirpalani et al (2006) <sup>95</sup> | Neonatal intensive care units (n=451) | Infants weighing <1000 g, less than full gestational age, and <48 hours old at the time of enrollment | <u>Liberal</u> : Algorithm of high transfusion thresholds                            | 5.7 (5.0)                       | 95%                       | Week 1: 14.9 (1.7) g/dL<br>Week 2: 13.1 (1.3) g/dL<br>Week 3: 12.0 (1.5) g/dL<br>Week 4: 11.2 (1.3) g/dL<br>Discharge: 10.8 (1.6) g/dL | Combination of death or survival selected complications* = 69.7%       | 17.5%                          | —                                                                                                                                                                                                                                      | —                                                  |
|                                      |                                       |                                                                                                       | <u>Restrictive</u> : Algorithm of low transfusion thresholds                         | 4.9 (4.2)                       | 89%                       | Week 1: 14.3 (1.9) g/dL<br>Week 2: 11.9 g/dL<br>Week 3: 10.9 (1.5) g/dL<br>Week 4: 10.1 (1.2) g/dL<br>Discharge: 10.6 (1.8) g/dL       | Combination of death or survival selected complications* = 74.0%       | 21.5%                          | —                                                                                                                                                                                                                                      | —                                                  |
| Lacroix et al (2007) <sup>96</sup>   | Pediatric ICU (n=637)                 | Critically ill children with Hb <9.5 g/dL                                                             | <u>Liberal</u> : Threshold of 9.5 g/dL<br><u>Restrictive</u> : Threshold of 7.0 g/dL | 0.9 (2.6)<br>1.7 (2.2)          | 98%<br>54%                | Lowest Hb in ICU: 10.8 g/dL<br>Lowest Hb in ICU: 8.7 g/dL                                                                              | New or progressive MODS: 12%<br>New or progressive MODS: 12%           | 4% at 28 days<br>4% at 28 days | Nosocomial infections: 25%<br>Nosocomial infections: 20%                                                                                                                                                                               | ICU stay 9.9 (7.4) days<br>ICU stay 9.5 (7.9) days |

\*Primary outcome is death or survival with bronchopulmonary dysplasia, severe retinopathy, prematurity, or brain injury.  
 Hb = hemoglobin; Hct = hematocrit; MODS = multiple organ dysfunction; ICU = intensive care unit.

hypothesis-generating because the composite neurologic outcomes were not designated *a priori*,<sup>97</sup> apnea was assessed by an unblinded nurse<sup>97</sup> and the differences were small, and the large number of outcomes increase the risk of false-positive results.

In the second study, 451 infants weighing less than 1000 g, whose gestational age was less than 31 weeks, and who were less than 48 hours old were randomly assigned to receive transfusion at either a low or high transfusion threshold.<sup>95</sup> The thresholds used varied by respiratory support using a prespecified transfusion algorithm. The primary composite outcome was in-hospital death, severe retinopathy, bronchopulmonary dysplasia, or brain injury on cranial ultrasound. The mean hemoglobin concentration was ~1 g/dL different between groups and the number of RBC transfusions were not significantly different. There was no difference in the composite outcomes (low threshold, 74.0% vs high threshold, 69.7%). This trial did not confirm the hypothesis that restrictive transfusion is associated with brain injury in preterm infants.

The largest study involved 637 children admitted to pediatric ICUs.<sup>96</sup> When a 7-g/dL threshold was compared to a 9.5-g/dL threshold, the mean hemoglobin concentration was ~2 g/dL lower in the restrictive group. RBC units were transfused to 46% of patients in the restrictive group and 98% in the liberal group. The frequency of new or progressive multi-organ dysfunction (primary outcome) was 12% in both groups and there were no significant differences in any of the secondary outcomes including death, which occurred in 4% of both groups.

## Transfusion Guidelines

Since the National Institutes of Health Consensus Conference developed landmark guidelines for transfusion of blood components, many organizations have issued guidelines with the intent of aiding clinical decisions. The administration of RBCs is based on the balance of benefits, risks, and costs. Physicians making transfusion decisions are faced with massive amounts of sometimes conflicting information. Since 1983, concerns about the transmission of human immunodeficiency virus and other viruses through blood components have significantly modified both real and perceived risks and benefits. Many organizations responded by issuing clinical practice guidelines advocating a more restrictive approach to the use of allogeneic RBC units and other components.<sup>87</sup>

Recommendations varied among the guidelines. Only one set of guidelines recommended a transfusion trigger; six advocated a range of transfusion thresholds based on clinical judgment, one recommended using clinical judgment only, and five did not comment on either clinical judgment or a transfusion trigger. Because of the paucity of clinical trials, the guidelines relied heavily on expert opinion, which sometimes (particularly more recently) valued the risks of virus transmission through blood components above all other consequences of the transfusion decision.

The recently updated guidelines developed by the American Society of Anesthesiology are by far the most rigorously

developed and methodologically sound.<sup>98,99</sup> As with several other guidelines, the American Society of Anesthesiology task force advised against a transfusion trigger, although it did suggest transfusion is usually indicated if hemoglobin concentration is less than 6 g/dL and is usually unnecessary if the hemoglobin concentration is greater than 10 g/dL. In patients with hemoglobin concentrations between 6 and 10 g/dL, transfusion decisions should be guided by the patient's risk of or actual bleeding, volume status, cardiopulmonary reserve, and vulnerability to complications of inadequate oxygenation. A recently published guideline for perioperative blood transfusion and blood conservation in cardiac surgery emphasized combined use of multiple factors, including interventions to reduce blood loss and institution-specific blood transfusion algorithms.<sup>100</sup>

## Decision Making in Red Cell Transfusion

### Approach to Evaluating a Bleeding Patient

In the care of a bleeding patient, the physician must first determine the degree of blood loss and rate of bleeding. As part of the initial assessment, the health-care team must rapidly address the patient's "ABCs" (airway, breathing, circulation). Although blood loss usually leads to circulatory compromise, the severe form, termed hemorrhagic shock, often leads to airway and breathing concerns. Once airway and breathing have been assessed and cleared, assessment of the heart rate and blood pressure—in addition to a rapid scan of the scene and the patient—will reveal obvious threats such as dramatic blood loss. During the initial assessment, patients must be completely uncovered and rapidly examined to rule out rapidly bleeding wounds, obvious signs of massive bleeding such as a rapidly expanding abdomen in patients with a ruptured abdominal aortic aneurysm, or severe hematochezia in patients with severe lower gastrointestinal bleeding. The focus is on possible causes of bleeding and important comorbid conditions, such as cardiovascular disease that could exacerbate any injury. An emergency complete blood cell count, type and crossmatch, prothrombin time, and partial thromboplastin time should be obtained. From these data and the clinical examination, the source and rate of bleeding are estimated. A decision can be made about the rate of RBC unit transfusion as well as any curative and supportive measures. For patients with gastrointestinal bleeding, consultations may be requested from the gastroenterology and possibly surgery departments. In the care of patients with postoperative bleeding, measures such as angiography or exploratory surgery should be considered.

In patients with active bleeding, the hemodynamic status and need for emergency intervention must be determined. Crystalloid is administered to maintain intravascular volume. Red cell transfusions are administered rapidly to maintain adequate oxygen-carrying capacity. Clinical judgment is needed to 1) estimate how much more bleeding may occur and how much lower the hemoglobin level will decrease, and then 2) perform expectant transfusion. Vital signs are examined for a decrease



in blood pressure and for tachycardia. The patient is asked about symptoms that can result from anemia, including cardiac ischemia (chest pain), congestive heart failure (dyspnea, paroxysmal nocturnal dyspnea, edema), fatigue, dizziness, weakness, and orthostatic hypotension unresponsive to intravenous fluids. The hemoglobin level is measured at regular intervals.

It is important to determine the presence of coronary artery disease because results of studies with animals and with human subjects suggest that patients with coronary artery disease are less tolerant of anemia. Review of the medical history for angina, myocardial infarction, and CABG surgery is essential. The electrocardiogram is examined for evidence of old myocardial infarction or ischemic changes. The chest radiograph is examined for cardiomegaly and other changes consistent with congestive heart failure. Patients with a history of peripheral or cerebral vascular disease are more likely also to have asymptomatic coronary artery disease.

In the care of patients with moderate to severe blood loss that has abated, there is more time for complete assessment of the source of bleeding, development of a course of management, and thorough evaluation of the patient. After bleeding has stopped and equilibration has occurred, the hemoglobin level stabilizes and the nadir hemoglobin level can be determined.

### **Surgical Patient**

Patients undergoing major surgical intervention often experience hemodynamic instability and frequently require RBC unit transfusion as a consequence of considerable blood loss. Various aspects of transfusion management for surgical patients are discussed in Chapters 37 through 40. One of the goals of resuscitation is to maintain oxygen delivery well beyond the anaerobic threshold. Hemoglobin concentration should be maintained above a value that maintains adequate  $\text{DO}_2$  and minimizes exposure to allogeneic RBC transfusion. However, optimal intraoperative thresholds have not been established. Current methods of monitoring effective circulatory blood volume and utilization of oxygen at the tissue level have limitations. Therefore, maintenance of adequate hemoglobin level has been recommended.<sup>98</sup> Frequent measurement can assist the anesthesiologist in administering one RBC unit at a time when blood loss is predictable. Blood loss exceeding 20% of blood volume in a short time may necessitate transfusion of multiple RBC units as well as administration of plasma and platelets. Reliable intraoperative measurements can be performed easily at the point of care with hemoglobin measurements obtained with standard blood gas analyzers. However, the accuracy of this measurement is influenced by the amount of intravenous fluid administered by the anesthesiologist and the fact that insufficient time has passed for equilibration to occur. Therefore, expert clinical judgment is required.

Standard anesthetic practice mandates intraoperative monitoring of cardiac function with measurement of continuous electrocardiographic and noninvasive or invasive blood pressure monitoring. Both monitoring techniques are essential but are insensitive to significant blood loss. This can delay recognition

of significant anemia until late in the process. However, optimal intraoperative thresholds have not been established and there are limitations to the current methods of monitoring effective circulatory blood volume and utilization of oxygen at the tissue level. A patient can have profound anemia without appreciable changes in either measurement. More sophisticated and invasive monitoring of cardiac function includes measurement of right- and left-sided ventricular filling pressure by means of central venous pressure and pulmonary artery catheters. Both techniques provide estimates of the effective circulating blood volume and are frequently used in high-risk surgery and in ICUs. Repeated measurement of central pressure and fluid challenges provide much more reliable assessment of effective circulating blood volume than does an isolated single measurement. Continuous monitoring of cardiac output with either pulmonary arterial catheters or esophageal Doppler techniques can be performed in many centers. Most of these techniques are reliable and frequently are used intraoperatively and during critical illness.<sup>101</sup> However, current monitoring techniques have a number of major limitations in guiding transfusion requirements. Careful clinical observation in conjunction with the physiologic data provide many of the necessary tools in the diagnosis and detection of clinically important anemia. The more difficult task is knowing how best to administer RBC units, intravenous solutions such as colloids and crystalloids, and vasoactive drugs.

Few data exist on whether invasive monitoring and different treatment strategies cause more harm than good. Most experienced anesthesiologists and critical care physicians have developed an approach to management of blood loss and hemodynamic instability. There is still enormous practice variation in selecting specific therapies. Randomized clinical trials are needed to assist in clinical decision making.

### **Chronic Anemia**

In chronic anemia, there is time for compensation to develop and for careful observation of the patient. Anemia is associated with an increase in blood flow resulting from decreased viscosity, greater release of oxygen caused by higher levels of 2,3-DPG, and an increase in cardiac output. There is time to determine whether these physiologic events are clinically important in the individual patient and carefully determine whether the patient has symptoms. Besides cardiac symptoms (angina, dyspnea), anemia can lead to nonspecific symptoms, such as fatigue, weakness, dizziness, reduced exercise tolerance, and impaired performance of activities of daily living. There also may be time to implement alternative treatments to correct anemia, depending on the cause. Iron, vitamin  $\text{B}_{12}$ , and folate can be replaced. Erythropoietin can be administered. It is important to correct preoperative anemia because the hemoglobin level strongly correlates with the probability of transfusion.<sup>102</sup>

### **Transfusion Threshold**

The optimal threshold for transfusion is uncertain in most clinical situations. The only adequately powered randomized clinical

trial showed it is safe to withhold blood until the hemoglobin level decreases to less than 7 g/dL.<sup>84</sup> The study was performed in critically ill patients and should be used to guide therapy in this setting. However, these patients are unique in that they are extremely ill, monitored very closely, and usually bedridden. It is uncertain whether the results should be applied to other clinical situations, such as care of postoperative surgical patients. There are no other randomized trials large enough to adequately guide clinical decision making in settings other than ICUs. Clinically important outcomes such as functional recovery have not been the subject of large-scale studies. In addition, different transfusion thresholds have not been adequately examined in high-risk patients with acute coronary syndromes or in patients with other forms of severe heart disease.

In the absence of evidence, it is necessary to rely on clinical judgment. In the authors' view, in patients who are symptom-free or who do not have active ischemic heart disease, a transfusion trigger of 7 g/dL should be used. For patients with an acute coronary syndrome, perhaps a higher transfusion threshold should be used (~8-10 g/dL). Patients with symptoms of anemia should be transfused as needed. No set of guidelines applies to every patient. In the end, careful clinical assessment of each patient with thoughtful consideration of risks and benefits should guide the transfusion decision.

### Dose and Administration

In patients who experience acute blood loss, the rate of administration of RBCs is guided by the rate of bleeding and hemodynamic compromise. In the treatment of rapidly bleeding patients, RBCs should be given at the rate necessary to maintain oxygen transport while definitive therapy is instituted. Rates may range from 5 to 10 units over 10 to 15 minutes in patients who are exsanguinating to rates in the range of 1 unit every 2 to 4 hours (1 mL/kg/hour) in patients with severe left ventricular dysfunction. When blood loss is predictable and not massive, RBC units should generally be administered one at a time. In the care of patients with chronic anemia, enough blood should be given to control symptoms. In most adult patients, 1 RBC unit increases the hemoglobin level approximately 1 g/dL and hematocrit approximately 3%. Ordinarily, 1 RBC unit is given over 1 to 2 hours. After transfusion of each RBC unit, measurement of hemoglobin level is repeated, and the patient reassessed.

### Future

During the next decade, advances and risks may dramatically alter transfusion practice. Research is under way to develop safe red cell components. Further clinical trials should be performed to better describe the clinical effect of different transfusion strategies. It is possible that within the next one or two decades, a cost-effective oxygen carrier will be developed that will reduce the use of allogeneic RBC units.

## Summary

Red cell transfusion is an extremely common treatment throughout the world. Blood is administered to improve oxygen delivery. Patients compensate for anemia through increased cardiac output and through redistribution of blood flow to the cardiac and cerebral circulation. Particular attention must be paid to maintaining adequate oxygen delivery to the heart. Results of the one adequately powered clinical trial, performed with ICU patients, suggest that a hemoglobin level of 7 g/dL is a safe threshold for red cell transfusion. For patients with cardiovascular disease, a higher transfusion threshold should be considered. RBC units should usually be administered one at a time, and the patient and hemoglobin level reassessed after each transfusion. Transfusion decisions should consider the rate of bleeding, the presence of underlying medical problems, the hemodynamic status of the patient, the presence of symptoms, and whether anemia is acute or chronic.

## Disclaimer

J. Carson has disclosed a financial relationship with Ortho Biotech and P. Hébert has disclosed financial relationships with Amgen, Johnson & Johnson, and Novo Nordisk.

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## 10

## Platelet Production, Kinetics, and Hemostasis

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Megakaryocytopoiesis begins with the lineage commitment of hematopoietic stem cells (HSCs), and includes the proliferation, maturation, and terminal differentiation of megakaryocytic progenitors. Circulating levels of thrombopoietin (TPO), the primary growth factor for the megakaryocyte (MK) lineage, induce concentration-dependent proliferation and maturation of MK progenitors by binding to the c-Mpl receptor. Decreased platelet numbers result in increased free TPO levels, enabling the compensatory response of marrow MKs to increase platelet production. C-Mpl activity is orchestrated by a complex cascade of signaling molecules that drive MK proliferation and maturation. Mature MKs form proplatelet projections that fragment into circulating platelets. Newly developed thrombopoietic agents operating via the c-Mpl receptor have proven useful in clinical thrombocytopenia trials. This chapter reviews the regulation of megakaryocytopoiesis and platelet production in normal and disease states, and discusses new approaches to thrombopoietic therapy.

**Platelet Production****Megakaryocyte Development**

Megakaryocytes give rise to circulating platelets through commitment of the multipotent stem cell to the MK lineage, proliferation of those progenitors, and finally terminal differentiation of MKs. This process is characterized by DNA endoreduplication, cytoplasmic maturation and expansion, and release of cytoplasmic fragments as circulating platelets. Within the marrow, MKs are derived from HSCs, which evolve from the multipotential hemangioblast. The hemangioblast gives rise to all blood and blood vessel precursor cells. The HSCs, which give rise to the early common myeloid progenitors (CMPs), includes the multilineage (granulocyte, erythrocyte, MK, and monocyte)

colony-forming unit (CFU-GEMM). Erythroid and MK lineages arise from a common MK-erythroid progenitor (MEP) derived from the early CMP. CMP differentiation is orchestrated by molecular signals controlled by regulatory transcription factors. Two major transcription factors involved in CMP differentiation are GATA-1, which drives differentiation of MEP, and PU.1, which regulates granulocyte-monocyte precursors.<sup>1</sup> The down-regulation of PU.1 expression in the CMP is the first event associated with the restriction of differentiation to erythroid and MK lineages.<sup>2</sup> In response to environmental factors, cytokines, and chemokines, the bipotential MEP can develop into the highly proliferative, early MK burst-forming unit (BFU-MK), or the more mature smaller CFU-MK, both of which express CD34.<sup>3</sup> The proliferating diploid MK progenitors (megakaryoblasts) lose their capacity to divide, but retain their ability for DNA replication (endoreduplication) and cytoplasmic maturation.

**Megakaryocyte Maturation**

The hallmarks of MK maturation are endoreduplication (polyploidization) and expansion of cytoplasmic mass. Mature MKs give rise to circulating platelets by the acquisition of cytoplasmic structural and functional characteristics necessary for platelet formation and function.<sup>4,5</sup> MKs reach cell sizes of 50 to 100 microns in diameter, with ploidy ranges up to 128N.<sup>6,7</sup> Early megakaryoblasts have the highest nuclear/cytoplasmic ratio. These immature cells contain elevated RNA levels, prominent ribosomes, and rough endoplasmic reticulum; they also express platelet peroxidase, and contain  $\alpha$ -granules, dense bodies, and primitive demarcation membranes. As the MK matures, the polyploid nucleus becomes horseshoe-shaped, the cytoplasm expands, and platelet organelles and the demarcation membrane system are amplified.<sup>8</sup> This robust cytoplasmic mass forms proplatelet projections that give rise to newly circulating (reticulated) platelets.<sup>9</sup> While the large polyploid MKs are easily identified by morphology, the small MK progenitor and immature MK are more difficult to discern. Their identification is facilitated with the use of antibodies to major platelet membrane glycoproteins (GPs) including the integrin  $\alpha_{IIb}\beta_3$  (CD41a or GPIIb/IIIa complex), CD41b (GPIIb),

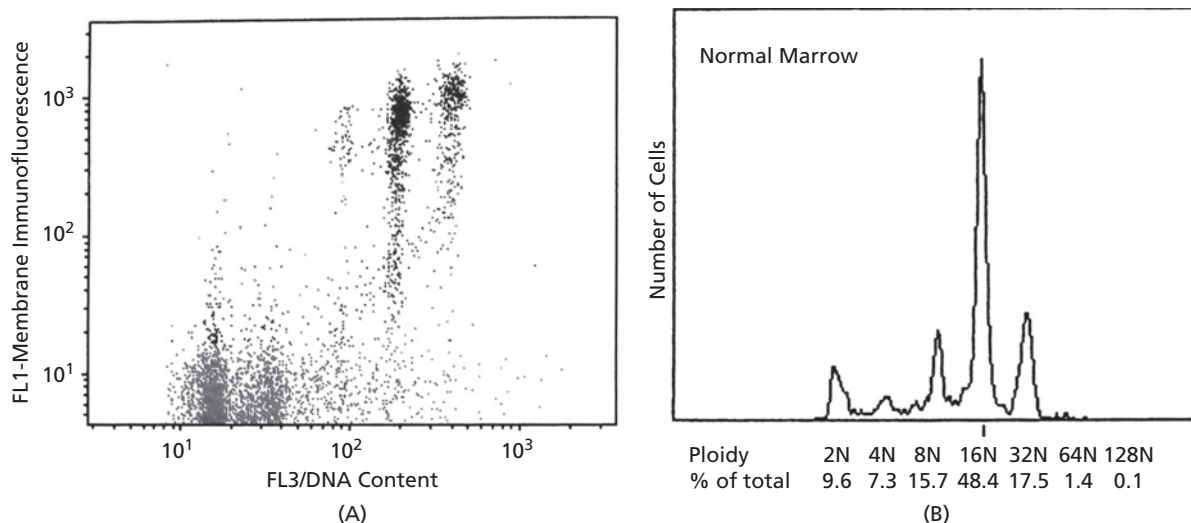


**Figure 10-1.** Ploidy distribution of normal human megakaryocytes (MKs) in unfractionated marrow using flow cytometry. (A) The distribution of marrow cells according to membrane-GPIIb/IIIa immunofluorescence and DNA content. Cells with background fluorescence represent the major marrow cell population with ploidy classes of 2N and 4N (bottom). The highly fluorescent cells represent the MK population with polyloid subclasses (top). (B) The DNA histogram of the MK population. The modal ploidy is 16N comprising about half of the MK population with approximately equal proportions of cells being  $\leq 8N$  and  $\geq 32N$ .

CD61 (GPIIIa), CD42a (GPIX), CD42b (GPIb), and CD51 (GPV), as well as to internal platelet  $\alpha$ -granule proteins, von Willebrand factor (vWF), platelet factor 4 (PF4),  $\beta$ -thromboglobulin ( $\beta$ -TG), fibrinogen, coagulation Factor VIII, and Factor V.<sup>10</sup> Immature 2N to 4N cells produce platelet peroxidase but have no detectable demarcation membrane or platelet  $\alpha$ -granules.<sup>8</sup> CD41 antibodies enable the detection of small “lymphoid” megakaryoblasts in marrow, as well as in CFU-MK by Day 4 or 5 of culture.<sup>11</sup>

Flow cytometry has been adapted to the direct analysis of human MKs in routine marrow aspirates.<sup>7,12</sup> Fluorescence-activated cell sorting has enabled the separation of pure ( $>98\%$ ) viable MK populations isolated on the basis of lineage markers. These cells respond to hematopoietic growth factors and synthesize both protein and DNA.<sup>6</sup> Moreover, flow cytometry has enabled the study of megakaryocytopoiesis in disease states, monitoring drug effects on human megakaryocytopoiesis, and studying the effect of hematopoietic growth factors on megakaryocytopoiesis in humans and primates.<sup>13–15</sup> Recent flow-cytometric studies have demonstrated that vWF is strongly expressed by early (2–4N) marrow MKs, enabling their complete resolution from other marrow cells at a level superior to that achieved with GPIIb/IIIa as a lineage-specific marker.<sup>10</sup>

Early MK differentiation is accompanied by the expression of GPIX, which exists as a complex with GPIb and GPV in the platelet membrane. In contrast, platelet GPIV and the thrombospondin (TSP) receptor (CD36) appear as late differentiation markers.<sup>16</sup> Following activation, platelet GPIIb/IIIa functions as a receptor for four adhesive proteins—fibrinogen, fibronectin, vitronectin, and vWF; however, GPIb is the major receptor for vWF,<sup>10</sup> as discussed later in this chapter.

The nuclear endomitotic cell cycle in MKs consists of a DNA replication S-phase, an M-phase with multiple pole spindles,

aborted anaphase B and cytokinesis, and a Gap-phase that enables reentry into the subsequent S-phase. Cyclin D3 is overexpressed in the G1-phase of maturing cells and is a key inducer of MK polyploidization.<sup>17</sup> Aurora-B/AIM-1, the fundamental regulator of mitosis, has normal localization and expression during prophase and early anaphase, but is absent or mislocalized in late anaphase MKs.<sup>18,19</sup> Chromosome segregation is asymmetrical with normal metaphase/anaphase checkpoints.<sup>20</sup> Ploidy classes are identified as geometric progressions of 2N (diploid), ie, 4N, 8N, 16N, etc.

The ploidy distribution obtained by flow cytometry in both fractionated and unfractionated normal human marrow demonstrates 16N in approximately half of the MK population, with the remainder of MKs split: 23% of cells being 8N or lower and 22% being 32N or higher<sup>12</sup> (Fig 10-1). The amount of surface GPIIb/IIIa, GPIIIa, GPIb, and CD36 correlates directly with cell size and ploidy. The mean diameter of MKs is  $37 \pm 4$  microns (mean  $\pm$  1 SD), compared with  $14 \pm 2$  microns for all marrow cells, and ranges from  $21 \pm 4$  microns for 2N cells to  $56 \pm 8$  microns for 64N cells.<sup>10</sup> The MK cytoplasmic mass is calculated as the product of the mean MK cytoplasmic volume and the total number of MKs.<sup>15</sup> By determining the ratio of MKs to nucleated erythroid precursors in marrow preparations by flow cytometry, the MK mass in patients with primary thrombocytosis was assessed.<sup>13,15</sup> Following treatment with anagrelide, both MK number and volume were decreased toward normal, resulting in decreased MK mass by about half, similar to the decrease in platelet count.<sup>15</sup> This finding was recently corroborated by in-vitro anagrelide studies.<sup>21</sup>

### Platelet Production and Release

During MK maturation, internal membrane systems, granules, and organelles are assembled in bulk. High-ploidy MKs form an extensive internal demarcation membrane, which is continuous

with the plasma membrane, and serves primarily as a reservoir for the formation of the precursor of cytoplasmic extensions called proplatelets. The open canalicular system, a channeled system for granule release and the dense tubular network, is formed before the initiation of proplatelet assembly.<sup>4</sup> Some platelet proteins, such as GPIb and GPIIb/IIIa, are synthesized and directed to the MK surface membrane, while other proteins are conveyed into secretory granules. Individual organelles migrate from the cell body to the proplatelet ends, with approximately 30% of the organelles/granules in motion at any given of time.<sup>5</sup> Fibrinogen is not synthesized but instead is taken up by MKs from the plasma through endocytosis and/or pinocytosis and selectively transferred to granules. Quantitative electron microscopic analysis has shown that MKs are located <1 micron from the marrow sinus wall, allowing rapid access of the newly formed platelets to the circulation.<sup>22</sup> Nascent platelets contain mitochondria and ribosomal RNA (the latter identifying them as reticulated platelets), as well as the components necessary for platelet hemostatic function. Platelets, however, do not contain nuclear material.<sup>4</sup>

Currently, there are two physical models of thrombopoiesis, which are not mutually exclusive. One model proposes platelet assembly and budding off the tips of proplatelets, which operate as assembly lines for platelet production.<sup>4</sup> The proplatelets extend into sinusoidal spaces, where they detach and fragment into individual platelets giving rise to about 2000 to 5000 new platelets/cell.<sup>23,24</sup> The other model of platelet biogenesis proposes that, within the MK cytoplasm, there are preformed territories with internal membranes demarcating prepackaged platelets, which are precipitously released upon fragmentation of the cytoplasm. In-vivo and ex-vivo observations of platelet release from MKs with phase contrast microscopy strongly support the explosive-fragmentation theory.<sup>25,26</sup>

### Regulation of Megakaryocytopoiesis

The processes of megakaryocytopoiesis and platelet production occur within a complex marrow microenvironment where chemokines, cytokines, and adhesive interactions play a major role.<sup>27</sup> Mechanisms regulating megakaryocytopoiesis operate at all levels of proliferation, differentiation, and platelet release.<sup>28,29</sup> In addition to steady-state megakaryocytopoiesis, which supplies  $10^{11}$  platelets every day and a new turnover every 7 to 10 days, MKs also respond to changes in requirements for circulating platelets, increasing more than 10-fold under conditions that demand platelet production.<sup>30</sup> Compensatory responses of marrow MKs increase platelet production through an increase in cell proliferation and maturation, and early release of platelets, which is evident within 24 to 48 hours after inducing thrombocytopenia. Following thrombocytapheresis and in thrombocytopenias caused by immune destruction, the marked increase in MK number and size is notable.<sup>7,31</sup> The normal frequency of human MKs in marrow is about one in 2000 nucleated cells. However, with a compensatory response, the number increases 10-fold<sup>32</sup> and the proportion of high-ploidy cells increases as

well.<sup>12</sup> Reciprocal decreases in MK size, ploidy, and volume occur with experimentally induced thrombocytosis.<sup>33,34</sup> These alterations, found in both experimental animals and human subjects, are primarily mediated by TPO.<sup>30</sup>

### Thrombopoietin

Thrombopoietin is the primary physiologic growth factor for the MK lineage. TPO also plays a central role in the survival and proliferation of HSCs.<sup>35,36</sup> TPO is the most potent cytokine for stimulating the proliferation and maturation of MK progenitor cells. It stimulates MKs to increase cell size and ploidy, and to form proplatelet processes that then fragment into single platelets.<sup>30</sup> In baboons, administration of recombinant human (rHu) TPO or a polyethylene glycol (PEG) derivative MK growth-and-development factor (PEG-rHuMGDF) produced a log-linear increase in marrow MK mass of up to 6.5-fold that was associated with a marked increase in cell ploidy. Concordantly, there was an increase in platelet count up to fivefold, reaching peak values after 2 to 4 weeks of TPO therapy (Fig 10-2). Because MK volume and ploidy attained predictable maximum values simultaneously, MK ploidy is an accurate measure of Mpl-ligand stimulation of megakaryocytopoiesis.<sup>13,14</sup> TPO can also act in synergy with other hematopoietic cytokines and has been used effectively to expand human HSCs and MK progenitor cells in vitro.<sup>35,37-39</sup>

TPO directly affects platelets as well; it enhances  $\alpha$ -granule secretion and aggregation induced by thrombin in a phosphoinositide-3 kinase (PI3K)-dependent fashion.<sup>40</sup> TPO also reduces the level of adenosine diphosphate (ADP), collagen, or thrombin necessary for aggregation,<sup>41,42</sup> and it stimulates platelet adhesion.<sup>43</sup> Thus, TPO affects mature platelets in a pro-aggregatory fashion.

Thrombopoietin is encoded by a single human gene, located on chromosome 3q26.3-3q27, which produces a 353-amino-acid precursor protein. The mature molecule, composed of 332 amino acids, is acidic and heavily glycosylated. Mutations (inversion or deletion) at its chromosomal locus are often found in megakaryocytic leukemia and other myeloproliferative disorders (MPDs) associated with thrombocytosis.<sup>44</sup> Mutations in regulatory regions of the TPO gene that result in overexpression of TPO and sustained intracellular signaling or disturbed regulation of circulating TPO cause familial essential thrombocythemia (ET) or familial thrombocytosis.<sup>30,45</sup> TPO shares high homology with erythropoietin (EPO) in its N-terminal half, reflecting a close evolutionary relationship between their receptor signaling pathways. TPO binds to the c-Mpl receptor on MKs and selectively initiates proliferation, maturation, and cytoplasmic delivery of platelets into the circulation.<sup>29,46</sup> TPO is produced constitutively by the liver and its circulating levels are regulated by the extent of binding to c-Mpl receptors on circulating platelets and marrow MKs, which results in the elimination of TPO-c-Mpl complexes.<sup>46,47</sup>

Messenger RNA specific for TPO is also found in the kidney, marrow stroma, and other tissues,<sup>48</sup> but the role of these tissues in megakaryocytopoiesis is unclear. Blood and marrow levels



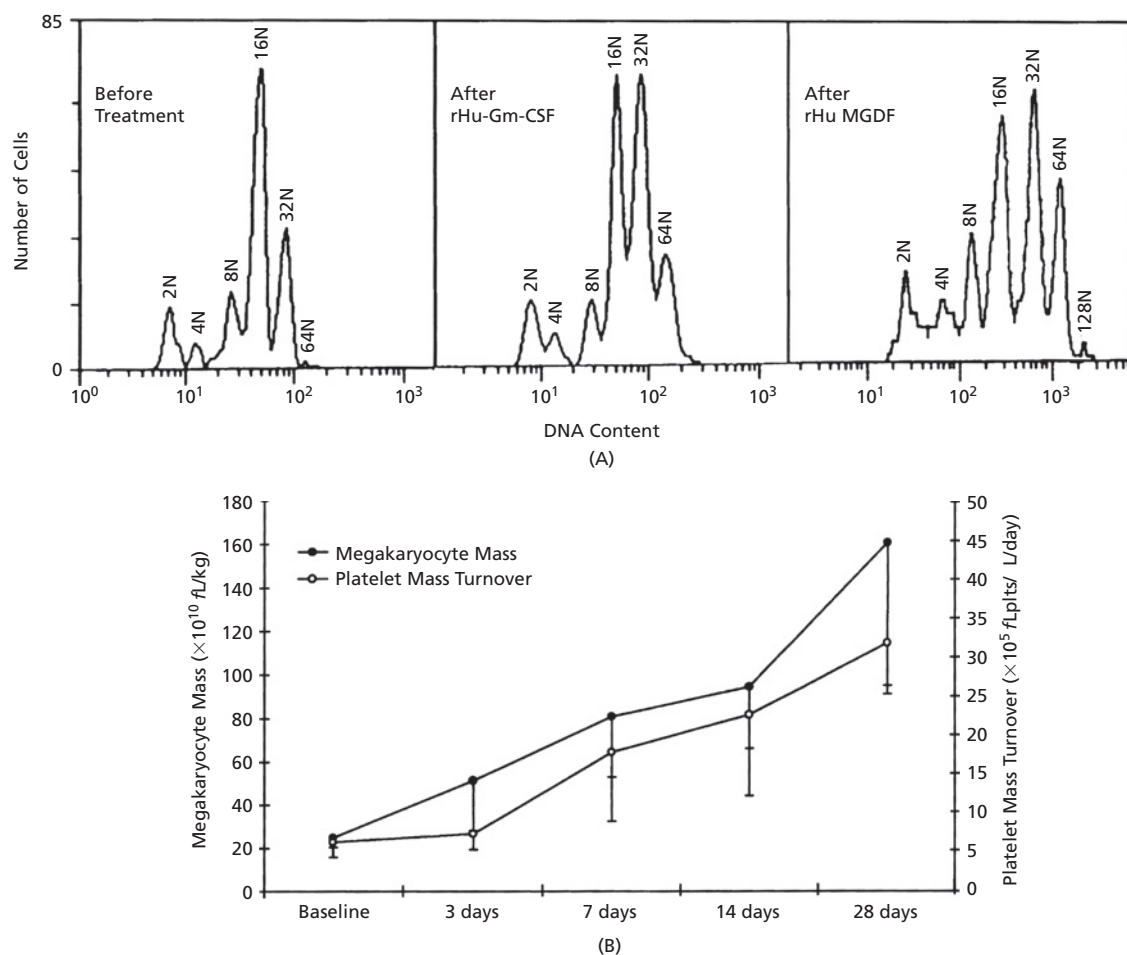


**Figure 10-2.** (A) Effect of in-vivo administration of megakaryocyte-stimulating factors on marrow megakaryocytes in nonhuman primates: ploidy changes in baboons. Left panel: before treatment. Middle panel: after treatment with recombinant human granulocyte-macrophage colony-stimulating factor (rHu-GM-CSF) or interleukin-6 for 8 days; there is a marked increase in ploidy. Right panel:

after treatment with recombinant human megakaryocyte growth and development factor (rHuMGDF) for 7 days; the increase in ploidy is more prominent than that observed with rHu-GM-CSF. (B) The effect of rHuMGDF on megakaryocyte mass and platelet production in nonhuman primates. A concordant increase in both measurements is evident over 2 to 4 weeks of therapy.

of TPO are usually inversely related to marrow MK mass and peripheral platelet counts.<sup>30</sup> Plasma levels of TPO (normally  $95 \pm 6$  pg/L) are increased by several orders of magnitude in patients with thrombocytopenia secondary to aplastic anemia or myelosuppression, and TPO levels decline after platelet transfusion or recovery of hematopoiesis.<sup>46,49</sup> However, in states of massive platelet destruction, such as immune thrombocytopenic purpura (ITP), TPO levels are not as escalated as might be expected from the degree of thrombocytopenia, probably due to the markedly increased platelet turnover rate.<sup>50</sup> Thus, megakaryocytopoiesis is regulated by plasma levels of unbound TPO, which reflects the balance between constitutive production and the rate of platelet and MK binding that is generally dictated by overall platelet production. Exceptions to steady-state platelet production occur during ET<sup>51,52</sup> or inflammation when circulating TPO levels are increased<sup>46,53,54</sup> because of enhanced hepatic expression driven by the acute phase protein, interleukin-6 (IL-6).<sup>55</sup>

### c-Mpl

The TPO receptor (c-Mpl) is predominantly expressed on heman-gioblasts, MKs at all stages of differentiation, and platelets. While receptor display is modulated by TPO binding and receptor internalization, c-Mpl is usually constitutively expressed. TPO avidly binds and activates the c-Mpl receptors on MK, which undergo conformational changes to initiate signal transduction. The JAK family of kinases constitutively bind to the membrane-proximal cytoplasmic domains of c-Mpl and initiate signaling. The activated JAK kinase also phosphorylates tyrosine residues within the receptor itself, as well as downstream signal transducers and activators of transcription (STATs), PI3K, and the mitogen-activated protein kinases (MAPKs). This cascade drives cell survival and proliferation, requires PI3K activation, and activates SHP1 and SHIP1 phosphatases and suppressors of cytokine signaling to limit cell signaling.<sup>35,36</sup> In humans, elimination of a functional c-Mpl gene (located on chromosome 1p34) or the congenital

absence of c-Mpl in children, results in severe thrombocytopenia caused by decreased hematopoietic stem and lineage-committed progenitor cells.<sup>30</sup> A recent study suggests that c-Mpl does not control HSC numbers, as had been previously argued, but rather facilitates the early expansion of differentiating clones.<sup>57</sup> Multiple mutations, including nonsense, frameshift, splice-site alteration, missense, or amino-acid substitutions, have been identified. These mutations result in a total absence of the c-Mpl receptor or functionally defective TPO-binding and/or signaling,<sup>58,61</sup> such as in congenital amegakaryocytic thrombocytopenia Type I (CAMT-I) in children, which is characterized by persistently low platelet counts  $<20,000/\mu\text{L}$  and progression to marrow failure and pancytopenia.<sup>58,62</sup> Hematopoietic stem cell transplantation (HSCT) is currently the only curative treatment.<sup>63</sup> In CAMT Type II, patients have more gradual development of pancytopenia caused by residual activity of the TPO receptor.<sup>60,64,65</sup>

An activating mutation within the transmembrane domain of c-Mpl has been reported to be associated with familial ET.<sup>66</sup> A c-Mpl polymorphism (Mpl Baltimore), with a single nucleotide substitution unique to individuals of African ethnicity (7% incidence of heterozygosity), is associated with reduced protein expression of Mpl, but a clinical phenotype of thrombocytosis.<sup>67</sup> A similar decrease in c-Mpl receptor on the cell surface is seen in patients with polycythemia vera (PV) and other MPDs. Thrombocytosis may therefore be related to the hypersensitivity to cytokines and signaling abnormalities found in these disorders.<sup>30</sup> Mutations in the JAK2 gene are prevalent in patients with MPD.<sup>68</sup> The molecular phenotype involving an activating JAK2 tyrosine kinase mutation, JAK2V617E, is a somatic point mutation causing the substitution of valine by phenylalanine in the protein product.<sup>69</sup> Because a cytokine receptor scaffold is necessary for the transforming and signaling activities of JAK2, mutations can drive proliferative disease only in cells that express Type I cytokine receptors. In PV, the mutational frequency can be as high as 97% and in ET up to 57%. The mutation is also expressed in other MPDs.<sup>70</sup> At diagnosis, ET patients with the mutation are older and have higher hemoglobin levels and white cell counts; the mutation also portends an increased mortality.<sup>71</sup> However, understanding the molecular basis of ET and other MPDs has introduced optimism for new therapeutic approaches to thrombocytopenia.

### Additional Growth Factors

Although TPO is the main physiologic regulator of megakaryocytopoiesis, it is also thought to operate in conjunction with other factors.<sup>72</sup> Other pleiotropic hematopoietic growth factors that stimulate MK growth alone or in combination with TPO include granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, IL-6, IL-11, stem cell factor, FLT ligand, fibroblast growth factor (FGF), and EPO.<sup>6,7,25,38,73,74</sup>

A novel MK growth-stimulating peptide (ARP26) derived from the cleavable C-terminus of the stress-associated form of acetylcholinesterase (AChE) has recently been described.<sup>75</sup> Stress-induced cholinergic signaling promotes

inflammation-associated thrombopoiesis. This peptide drives the proliferation of CD34+ hematopoietic progenitor cells and MKs in vitro, as well as MK progenitors in transgenic mice.

### Negative Regulators of Megakaryocytopoiesis

Several factors are known to inhibit MK development, including transforming growth factor-beta1,<sup>76</sup> PF4, and IL-4.<sup>77-78</sup> Src kinase inhibitors have also recently been shown to negatively regulate MK proliferation by inducing early MK differentiation and functional platelet-like fragment formation in vitro.<sup>79,80</sup> This important discovery may enable new therapeutic modalities for treating thrombocytosis. A recent study reported that the Rho/ROCK pathway, a regulator of the actin cytoskeleton, may act as a negative regulator of proplatelet formation.<sup>81</sup>

### Cellular Interactions and Chemokines

Megakaryocytopoiesis and thrombocytopoiesis also require cellular interactions between HSCs and marrow stromal cells.<sup>27</sup> Stromal-derived factor-1 (SDF-1) of the CXC family is the key chemokine involved in the retention of hematopoietic precursor cells in the marrow. SDF-1 supports megakaryocytopoiesis and homing of HSCs to the marrow during fetal development.<sup>82</sup> SDF-1 is produced locally by stromal cells and promotes the migration and contact of immature MKs with a permissive, endothelial-enriched marrow environment<sup>27,83</sup> in an  $\alpha 4\beta 1$ -dependent manner.<sup>84</sup> FGF-4 fortifies the adhesion of MK progenitors to marrow endothelial cells, supporting survival and maturation, and SDF-1 enhances the movement of MKs to the junctions between sinusoidal endothelial cells via vascular endothelial cadherin, thus driving thrombopoiesis.<sup>27</sup> The SDF-1 receptor, CXCR4, is expressed along the entire MK differentiation pathway from early progenitors to platelets.<sup>82</sup> CXCR4 signaling in MKs involves the serine/threonine MAPKs, MAPK, and AKT MAPK pathways. These, in turn, include extracellular signal-related kinase (MEK) and extracellular signal-regulated protein kinase (ERK)1/ERK2, to regulate cell proliferation and differentiation. Upregulation of the CXC cytokine, CTAP III, in MKs is associated with maturation and, as with other chemokines, is involved in cellular interactions with extracellular matrix and platelet production.<sup>85</sup>

### Nuclear Transcription Factors

Megakaryocyte development and thrombocytopoiesis are controlled by the concerted action of transcription factors, which form complexes that coordinately regulate chromatin organization to specifically activate the genes of MK lineage precursors and/or concurrently repress genes that support other cell types. Many MK-specific genes are coregulated by GATA and friend of GATA (FOG) together with acute myeloid leukemia/runt-related transcription factor 1 (AML/RUNX-1) and ETS proteins. Mutations in transcription factor genes result in the translation of aberrant proteins that may cause thrombocytopenia or thrombocytosis.

Megakaryocytic and erythroid lineages, which are derived from a common bipotential progenitor, share many early

lineage-restricted transcription factors.<sup>86</sup> The zinc-finger protein GATA-1 is the principal transcription factor directing MK development by forming complexes with other transcription factors (FOG, ETS, and RUNX-1).<sup>87</sup> One of the initial events during MK/E lineage restriction is downregulation of PU.1, the main transcription factor responsible for myeloid cell differentiation,<sup>1</sup> and upregulation of GATA-1. Reciprocal antagonism of PU.1 and GATA factors balances lineage commitment decisions.<sup>88,89</sup> GATA-1 mutations lead to severe diseases involving both erythroid cells and MKs.<sup>90,91</sup> A shorter isoform of GATA-1, GATA-1s, is considered to be an early stimulus in the leukemogenesis of trisomy 21.<sup>91,92</sup> The association of the N-terminal finger of GATA-1 with its cofactor, the nine-zinc finger FOG-1, is critical for embryonic hematopoiesis and MK development.<sup>93</sup>

RUNX-1 (or AML-1) is essential for generation of all definitive hematopoietic lineages. The RUNX-1/AML-1 core binding factor (CBFb) complex binds the N-terminal transcription activation domain of GATA-1, which enables the programming of MK lineage commitment.<sup>94</sup> Chromosomal translocations in the *RUNX-1* gene are frequently associated with leukemia, eg, the t(8;21) in AML resulting in a dominant-negative RUNX-1-ETO fusion protein. Germline mutations of RUNX-1 are found in the autosomal dominant familial platelet disorder with multiple platelet defects, reduced c-Mpl, and predisposition to AML.<sup>95</sup>

The ETS family of factors resides in close proximity to GATA sequences in MK-specific promoters.<sup>96</sup> Friend leukemia integration-1 (Fli-1), located on chromosome 11q24, is an ETS factor expressed in primary MKs and hemangioblasts. Its loss is associated with the Jacobsen/Paris-Trousseau syndrome<sup>97</sup> and thrombocytopenia with large fused granules within abnormal platelets.<sup>98</sup> Finally, nuclear factor erythroid 2 (NF-E2) controls terminal MK maturation, proplatelet formation, and platelet release<sup>89,99</sup> by regulating a panoply of MK genes that are crucial elements in the processes of platelet production.

### Clinical Use of Thrombopoietic Agents

It was expected that physiologic and powerful stimulators of thrombocytopoiesis, rHuTPO or the truncated cloned rHuMGDF, would be effective in reversing thrombocytopenia caused by myelosuppression. In nonhuman primates, administration of rHuTPO or PEG-rHuMGDF resulted in markedly positive responses<sup>13,14</sup> (Fig 10-2). However, in early human trials, cross-reacting neutralizing antibodies to endogenous TPO developed, which led to thrombocytopenia in some cases and the cessation of these trials.<sup>56,100</sup> In later studies, rHuTPO was used to increase the yield of plateletpheresis for transfusion purposes.<sup>101</sup> However, administration to patients following aggressive chemotherapy showed no positive effect on platelet recovery.<sup>102</sup> In addition, the administration of rHuTPO before or after stem cell transplantation and myeloablative therapy had no impact on the duration of severe thrombocytopenia, nor did it reduce the requirement for platelet transfusions after transplantation or in AML patients after chemotherapy.<sup>103-105</sup> The failure of escalated TPO levels to stimulate platelet production in these

circumstances may be related to the paucity of the marrow MK progenitor population.<sup>47,106</sup>

In a Phase I study by the Children's Oncology Group, Children's National Medical Center, children with solid tumors received rHuTPO with granulocyte colony-stimulating factor (G-CSF) after combination chemotherapy. Time to hematologic recovery and the median number of platelet transfusions were somewhat improved compared with historical controls receiving combination chemotherapy with G-CSF alone.<sup>107</sup> A recent review of thrombopoietic factors in chronic marrow failure suggests that, although the temporal pace of response appears to be relatively slow, durable responses can be achieved in patients given thrombopoietic factors for extended periods.<sup>108</sup> Thus, TPO may prove useful in thrombocytopenic states where stem cells have not been severely depleted and the marrow microenvironment is healthy enough to support thrombopoietic activity.

Human trials of therapy for thrombocytopenia have also employed the pleiotropic cytokines, IL-1, IL-3, and IL-6, but their clinical development was abandoned because of limited effectiveness or excessive toxicity.<sup>109</sup> The multilineage cytokine IL-11 has been shown to attenuate chemotherapy-induced thrombocytopenia, and IL-11 (Neumega, Wyeth Pharmaceuticals, Collegeville, PA) is currently the only drug approved by the US Food and Drug Administration (FDA) for treating thrombocytopenia. However, IL-11 has limited effectiveness with no clear cost benefit over platelet transfusion.<sup>108</sup> A new generation of thrombopoietic agents have invigorated the field, however, with positive clinical results reported in patients with ITP and other disorders.<sup>56</sup>

AMG531 is an engineered TPO peptide mimetic composed of a recombinant protein carrier Fc domain linked to multiple Mpl-binding domains, which affects megakaryocytopoiesis similarly to TPO.<sup>110</sup> In Phase I and II studies of ITP patients, 7 of 12 and 12 of 16 patients had a doubling of their platelet counts and a rise to >50,000/ $\mu$ L. In Phase II, a weekly injection was given for 6 weeks; average peak platelet counts were 135,000/ $\mu$ L and 241,000/ $\mu$ L, for 1  $\mu$ g/kg and 3  $\mu$ g/kg, respectively.<sup>111</sup> More than 100 patients from these initial AMG531 studies have enrolled in an open-label study of long-term treatment for ITP. Interim analysis of 36 patients, most treated with a stable weekly dose for more than 48 weeks, showed a consistent mean platelet count greater than 100,000/ $\mu$ L. Discontinuation of therapy has resulted in the return of platelet counts in all subjects to baseline. It is noteworthy that in all three studies, AMG531 was well tolerated, and the major reported side effect was mild headache. However, increased marrow reticulin formation has been identified in two patients, and this finding will need to be closely followed.

Eltrombopag is a small-molecule (546 D), nonpeptide TPO-receptor agonist<sup>110,112-114</sup> available in an oral preparation. It uses JAK2 and STAT signaling pathways to cause human CD34+ cells to differentiate into megakaryocytes and produce platelets.<sup>113</sup> In a Phase I trial of 73 healthy subjects, one daily oral dose given for 10 days produced a dose-dependent rise in platelet count up to 1.5-fold over baseline. For the highest dose, the rise started on Day 7 and peaked at Day 16, without

reported side effects. Eltrombopag has shown significant activity in ITP patients. In a randomized, double-blind, placebo-controlled Phase II trial, ITP patients were given daily oral treatment for 6 weeks.<sup>110</sup> Platelet counts rose to  $>50,000/\mu\text{L}$  in up to 86% of patients. The median peak platelet counts were  $183,000/\mu\text{L}$  compared to  $16,000/\mu\text{L}$  in the placebo group. As with AMG531, cessation of eltrombopag treatment caused the platelet count to return to baseline in most patients. No adverse effects were reported.

In a randomized Phase II trial,<sup>115</sup> thrombocytopenic patients with chronic hepatitis C were treated for 4 weeks with placebo or escalated daily doses of eltrombopag. The median platelet count increased to  $209,000/\mu\text{L}$  in patients at the highest dose. No worsening of liver function was reported. Following this phase, patients were eligible to undergo interferon treatment along with open-label eltrombopag.

A Phase I trial with a long-acting pegylated TPO-mimetic peptide (peg-TPOmp), was described<sup>116</sup> in which 40 volunteers were randomly assigned to receive this TPO receptor agonist or placebo as a single intravenous bolus. Platelet counts increased dose-dependently, reaching peak levels at Days 10 to 12, and returned to baseline within 3 to 4 weeks. Platelet function was normal, and no serious adverse events or detection of antibodies against peg-TPOmp were reported.

A minibody agonist of c-Mpl was recently used in a novel therapeutic approach to treat thrombocytopenia.<sup>117</sup> IgG antibodies were engineered against the TPO receptor with absent or very weak agonist activity; these agonist minibodies, which include diabody or single chain (Fv) 2 [sc (Fv) 2], were as potent as the natural ligand. Two minibodies were successfully constructed to bind and activate two mutant types of dysfunctional Mpl receptor that cause CAMT. Another TPO-agonist antibody (MA01G4G344) showed stimulation of a TPO-dependent cell line and increased human MK-CFCs in vitro. A single injection increased the platelet count in human-c-Mpl transgenic mice for  $>1$  month.<sup>118</sup>

New synthetic c-Mpl agonists under preclinical development have recently been described. One is an orally active, nonpeptidyl TPO receptor agonist, AKR-501 (formerly YM 477).<sup>119</sup> In-vitro AKR-501 stimulated the growth of TPO-dependent cell lines and increased growth of megakaryocytes from human CD34+ cells. Its in-vivo effect was examined in mice producing platelets from human HSCs after transplantation. Oral administration of AKR-501 showed a dose-dependent increase in the number of human platelets, up to 3.0-fold by Day 14. Oral doses have been tested in healthy human subjects,<sup>110</sup> elevating the platelet count up to 2.8-fold without adverse effects. Another synthetic c-Mpl agonist, NIP-004, induced the proliferation of various TPO-responsive cell lines, primary human hematopoietic progenitor cells, and human thrombocytopoiesis in a xenotransplant animal model; the latter showed a 1.5-fold increase of megakaryoblasts, a 3.0-fold increase of mature polyploid MKs, and a 6.0-fold increase in human platelets.<sup>120</sup> Similarly, SB-497115 is a nonpeptidyl TPO-R agonist that increased platelet counts in a dose-dependent fashion when administered orally to healthy volunteers.<sup>121</sup> It is

important to note that the efficacy and safety of all of these molecules in clinical settings have yet to be determined.

## Platelet Kinetics

### Platelet Production

Circulating platelets are anucleate cells between 2 and 4 microns in diameter with a volume of 6 to 11 fL. The normal platelet count ranges from 150,000 to 450,000/ $\mu\text{L}$ . The overall rate of platelet production under steady-state conditions in healthy humans ranges from 35,000 to 44,000 platelets/ $\mu\text{L}/\text{day}$ .<sup>122-126</sup> Steady state assumes that platelet removal is equivalent to platelet production when the platelet count is constant. As expected, the MK mass in a healthy marrow generally correlates directly with platelet turnover.<sup>13,14,127</sup> However, intramedullary destruction of platelets can occur or abnormalities of megakaryocytopoiesis can result in impaired transformation of the cytoplasmic MK mass to circulating viable platelets. This discrepancy detected between megakaryocytic mass and platelet turnover is called *ineffective thrombocytopoiesis*. Therefore, the reliability of platelet turnover estimates depends on the accuracy with which each of the three variables used in calculating platelet turnover can be determined—mean platelet life span, recovery of platelets in the circulation, and blood platelet count. The summation error may be considerable, as occurs among patients with ITP.<sup>124</sup>

Newly released reticulated platelets (RPs) are identified by their increased RNA content<sup>128</sup>; murine studies have directly confirmed that RPs are the platelet subset most recently released from megakaryocytes into the blood.<sup>129,130</sup> The RP% in humans correlates with thrombopoietic activity.<sup>131</sup> Analogous to red cell reticulocytes, RPs increase in response to destructive thrombocytopenia and decrease when megakaryocyte hypoproliferation occurs with chemotherapy or intrinsic marrow aplasia.<sup>132</sup> The absolute RP count in humans with normal platelet counts ranges from 15,000 to 48,000/ $\mu\text{L}$ ; this average platelet production agrees with isotopic labeling studies. RNase-based RP analysis allows for a protocol-defined standardized RP% measurement independent of platelet size<sup>133</sup> and, therefore, is amenable to inter-laboratory use.<sup>134</sup> Determination of the RP maturation index or immature platelet fraction further refines the sensitivity of RP detection of early increases in platelet production, which may predict recovery from chemotherapy-induced thrombocytopenia and independence from transfusion therapy.

### Platelet Distribution

In humans, approximately two-thirds of platelets are in the general circulation. The remaining platelets are reversibly sequestered, primarily in the spleen. The distribution between the general and splenic pools of platelets can be determined by measuring the proportion of radiolabeled platelets remaining in the general circulation after infusion. The size of the splenic pool has been estimated to be approximately 30% of whole-body platelet mass.<sup>122,123,126,135,136</sup> In persons without a spleen, nearly 100% of

infused platelets are recovered. In patients with splenomegaly, by contrast, as much as 90% of total-body platelet mass can be sequestered in the spleen.<sup>136</sup>

It is currently controversial whether splenic pooling alters platelet survival. Splenectomy has been shown in some studies to cause prolongation of platelet life span,<sup>137</sup> while other studies found no change in platelet survival after splenectomy.<sup>138</sup> However, data from an animal model demonstrated a significant (>40%) increase in platelet life span, after splenectomy.<sup>139</sup> Platelet accumulation in the spleen is rapid and reaches 90% of maximum activity within approximately 12.5 minutes after injection of labeled platelets.<sup>123,135</sup> Although the role of the spleen in the regulation of the platelet count is controversial, splenic pooling of platelets is itself reversible. For example, intravenous epinephrine, which reduces blood flow to the spleen and causes the organ to empty passively into the circulation, normally causes the platelet level to increase by 30% to 50%, but does not affect platelet concentration in asplenic individuals.<sup>136</sup> Massive splenomegaly results in sequestration of as much as 90% of the total platelet population within the enlarged spleen.<sup>140</sup> In patients with more moderate splenomegaly, such as seen in patients with cirrhosis and portal hypertension, splenic sequestration is less, and platelet counts generally range from 60,000 to 100,000/ $\mu$ L. Hepatic pooling of infused platelets is also significant initially, reaching a maximum of approximately 16% of whole-body dosing 6 to 8 minutes after injection of radiolabeled platelets. This activity reaches a steady state after approximately 45 minutes. At equilibrium, hepatic platelet activity accounts for approximately 10% of total-body platelets.<sup>123,135</sup> This percentage of hepatic pooling may be increased after the spleen is removed.

### Platelet Survival

The finite platelet life span in healthy persons is  $9.5 \pm 0.6$  days,<sup>122</sup> and platelet disappearance is generally linear, primarily reflecting platelet senescence.<sup>125</sup> However, in patients with consumptive thrombocytopenia, the platelet life span is shorter, and the disappearance pattern becomes exponential, primarily reflecting random platelet removal.<sup>141,142</sup> Platelet survival time has been shown to shorten progressively as the platelet count decreases to  $<100,000/\mu$ L.<sup>125,143</sup> Therefore, a shortened platelet survival time in patients with thrombocytopenia may not necessarily indicate a pathologic destructive process. Hanson and Slichter<sup>125</sup> proposed a model for platelet removal that predicted shortening of platelet survival time in relation to the level of thrombocytopenia. The analysis indicated that 82% of platelet turnover in healthy persons is caused by platelet senescence, and approximately 18% is caused by a fixed requirement of platelets to support vascular integrity. Hemostatic loss represents about 7000 to 10,000 platelets/ $\mu$ L/day; loss of platelets for hemostatic integrity appears to be a fixed absolute number. Thus, in thrombocytopenia caused by marrow hypoplasia, the daily proportion of platelets removed by hemostatic consumption rises dramatically.<sup>144</sup> When platelet life span measurements have been prospectively studied in patients with moderate to severe thrombocytopenia

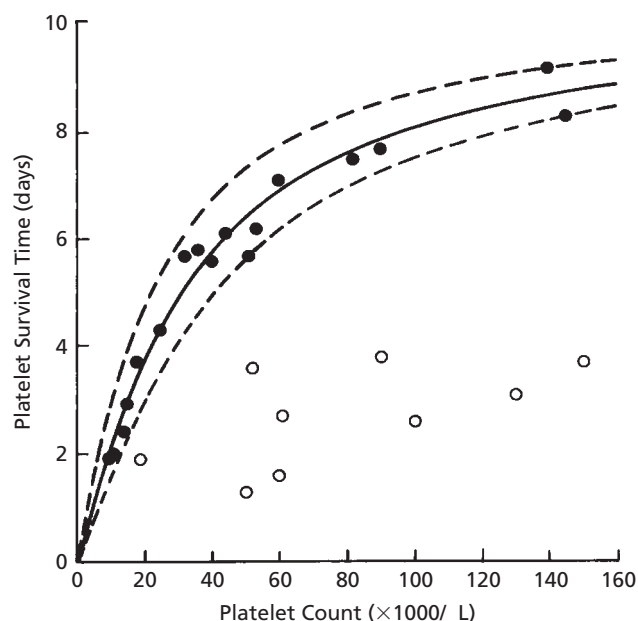


**Figure 10-3.** Relationship between survival time of  $^{111}\text{In}$ -oxine-labeled autologous platelets and circulating platelet count. *Closed circles* indicate patients with megakaryocytic hypoplasia. *Open circles* represent patients with immune thrombocytopenia (ITP). *Solid line* represents the best fit-to-data obtained with the first patient group; therefore, platelet survival could be predicted on the basis of the platelet count in these patients. All data points contained within the *dashed lines* were consistent with a value of random platelet turnover that ranged from 15.1% to 28.0% of overall platelet turnover.

(10,000 to 150,000/ $\mu$ L),<sup>144</sup> the results in patients with megakaryocytic hypoplasia are predicted by the platelet count (Fig 10-3). All data points were consistent with values of platelet turnover that ranged from 15.1% to 28.0% of overall platelet turnover. In contrast, platelet life span in patients with ITP was much shorter than the value predicted by the platelet count.

The physiology of senescent platelet removal is postulated to include irreversible changes in membrane glycoproteins; increased platelet-associated immunoglobulin (with subsequent clearance of those high-immunoglobulin-expressing platelets by splenic macrophages); increased exposure of the negatively charged procoagulant lipid, phosphatidylserine, on the external platelet membrane; and decreased levels of surface sialic acid.<sup>145</sup> Not surprisingly, the RP% has proven valuable for detecting changes in overall platelet turnover. The RP% has been applied for predicting thrombopoietic recovery, transfusion independence, and thrombotic risk.

The latter has been demonstrated in several settings. An increase in the RP% was associated with subsequent graft-vs-host disease after allogeneic HSCT.<sup>146</sup> Similarly, an asymptomatic increase in the RP%, with no change in platelet counts, from the first to second trimester in pregnancy was associated with subsequent preeclampsia.<sup>147</sup> Asymptomatic patients with steady-state thrombocytosis and an elevated RP% or absolute RP count were at increased risk for subsequent thrombosis,<sup>148</sup> and

their relative risk was higher than the risk associated with a high platelet count alone. The RP% and absolute RP count in thrombocytosis decreases with either successful aspirin therapy<sup>149</sup> or hydroxyurea.<sup>150</sup>

A compensatory increase in platelet production and the corresponding rise in circulating RP% are well established in patients with ITP, in whom antibodies targeting platelet membrane epitopes<sup>151</sup> cause platelet clearance via splenic macrophage Fc receptors.<sup>152</sup> Platelet life span in severe ITP can be shortened to hours. Although the marrow can increase megakaryocyte mass by 10-fold,<sup>153</sup> this is insufficient to maintain a normal platelet count with ITP; the increased RP% confirms the compensatory marrow production response. Once successful therapy (steroids, intravenous immune globulin, Rh Immune Globulin therapy)<sup>154</sup> for ITP has been instituted, the RP% has been shown to decline before platelet count recovery. The RP% continues to decrease as the platelet count approaches the normal ranges, indicating that restoration of a normal platelet life span rapidly downregulates RP release from MKs.

However, even patients with severe ITP appear to have relatively normal hemostasis,<sup>155</sup> and most ITP patients, especially children, do not have significant blood loss despite very low platelet counts. One reason may be that overall circulating platelet function is preserved because of the higher proportion of robust RPs. In human studies of thrombocytopenia, younger platelet cohorts had greater aggregation and adherence responses compared with older platelet subsets.<sup>156</sup> In animals, RPs have a lower threshold for  $\alpha$ -granule release than older platelets.<sup>157</sup> By contrast, in human ITP,<sup>158</sup> RPs do not appear to have a lower threshold for  $\alpha$ -granule release; instead, RPs have twice as much  $\alpha$ -granule content as older circulating platelets. RPs also have an increased density of adherent membrane receptors<sup>159</sup> than older cohorts, perhaps accounting for the lesser extent of bleeding in ITP compared to chemotherapy patients with similar platelet counts.

### Platelet Destruction

When platelets disappear from the circulation, they are mostly taken up by the mononuclear phagocytic system.<sup>135,143</sup> It is evident that the spleen and liver are the major sites of removal of platelets; after injection of <sup>111</sup>In-labeled platelets, radioactivity in the liver increases significantly and linearly with time from a level of 9.6% at equilibrium to 28.7% of total-body radioactivity during clearance.<sup>123,135</sup> Mean splenic radioactivity increased from 31.1% to 35.6% during clearance of radiolabeled platelets. After labeled platelets are removed from circulation (approximately 10 days after administration), 30.3% of whole-body platelet radioactivity is neither in the liver nor in the spleen. Platelets not removed by these two organs are presumably removed by the mononuclear phagocytic cells of the marrow.<sup>160</sup>

### Platelet Kinetics and Transfusion Therapy

As mentioned previously, thrombocytopenia causes the fixed daily requirement of platelets needed to maintain hemostasis to increase to a greater proportion (>60%)<sup>144</sup> of overall

platelet turnover.<sup>125</sup> Platelet kinetics studies<sup>125,144</sup> have predicted that transfusion of sufficient platelets to increase the circulating count from the 10,000/ $\mu$ L range to the 100,000/ $\mu$ L range should result in prolongation of platelet survival from 2 to 3 days to 8 to 9 days. The required frequency of platelet transfusion consequently would be decreased. This conclusion was corroborated by a prospective study of acute leukemia patients, who were stratified to receive escalated doses of platelets, ranging from  $4 \times 10^{11}$  to  $>8 \times 10^{11}$ . There was a direct, linear correlation between platelet dose and both platelet increment and transfusion-free interval.<sup>161</sup>

Another prospective, randomized, blinded, cross-over trial comparing low- and high-dose plateletpheresis products also confirmed the benefits of an increased platelet dose.<sup>162</sup> Forty-six patients undergoing HSCT were each given 79 paired transfusions, with low-dose ( $3.1 \times 10^{11}$ ) and high-dose ( $5.0 \times 10^{11}$ ) products in random sequence. Mean increments of 17,000/ $\mu$ L were seen with the lower dose, compared to 31,000/ $\mu$ L with the higher dose; a significant prolongation in the interval between platelet transfusions was also seen with the latter. In nonrandomized studies, transfusion of apheresis products with higher platelet content to thrombocytopenic patients has led to significantly higher increments and longer intervals between platelet transfusions.<sup>163</sup>

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## Platelet Hemostasis

The platelet is central to cell-based hemostasis. Platelet membrane receptors mediate binding to endothelium and sub-endothelial sites of damage, and this adhesion triggers transmembrane signaling, platelet activation, and procoagulant function, including release of procoagulant granule contents and exposure of internal membrane phospholipids. The procoagulant surface of the platelet causes assembly of the coagulation cascade, leading to thrombin formation, which, in turn, amplifies the procoagulant response and produces fibrin within the platelet matrix for superior hemostasis. Clot consolidation and protection from fibrinolysis are further augmented by platelet release of Factor XIII and PF4 into the clot milieu (Table 10-1). The following section details the physiology of platelet hemostasis and interaction with soluble coagulation factors, congenital and acquired platelet defects and their clinical phenotype, and the function of transfused platelets.

### Platelet Interaction with Blood Vessels

The vascular endothelium maintains a default anticoagulant surface by secreting factors that block soluble coagulation activity, including heparan sulfates, thrombomodulin, and tissue plasminogen activator. Antiplatelet factors are also intrinsic to the healthy endothelial cell lining; these include a net negative surface charge to repel similarly charged platelets, release of nitric oxide and prostacyclin to inhibit platelet aggregation, and surface expression of ADPase to inactivate platelet-Dreleased factors ADP.<sup>164,165</sup> The platelet-vessel wall interaction at the high flow

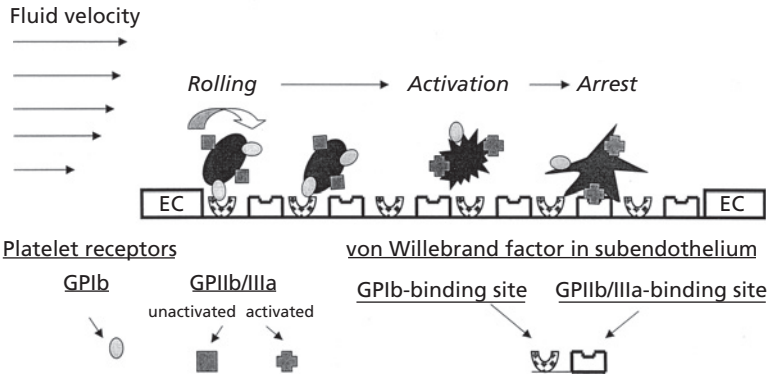


**Figure 10-4.** Platelet adhesion to subendothelial von Willebrand factor (vWF) in flowing blood. Endothelial injury exposes subendothelial collagen, which binds vWF and presents vWF attachment sites to platelets streaming along the vessel wall. Platelet GPIb rapidly attaches to its binding site on vWF, but this adhesion is short-lived, resulting in platelet rolling in close proximity to the subendothelium. The vWF-GPIb interaction causes transmembrane signaling, activating the platelet, and conformationally transforming platelet GPIIb/IIIa to avidly bind the RGD domain on vWF. This secondary adhesion is essentially irreversible, resulting in anchoring of the platelet to the exposed subendothelium, and allowing subsequent propagation of a platelet-fibrin clot.

**Table 10-1.** Platelet Prothrombotic Functions

|                                                                                  |
|----------------------------------------------------------------------------------|
| <b>• Membrane Components</b>                                                     |
| Thromboxane A <sub>2</sub> formation (cyclooxygenase-dependent)                  |
| Phosphatidylserine expression (translocation from the internal membrane surface) |
| <b>• Platelet Microparticles</b>                                                 |
| <b>• Receptor:Ligand Interactions Promoting Adhesion or Activation</b>           |
| GPIb/IX:vWF                                                                      |
| GPIIb/IIIa:fibrinogen and GP IIb/IIIa:vWF                                        |
| GPIa/IIa:collagen                                                                |
| GPIb:thrombin                                                                    |
| GPVI:collagen                                                                    |
| PAR-1:thrombin                                                                   |
| α <sub>2</sub> -Adrenergic receptor:epinephrine                                  |
| ADP receptor:ADP                                                                 |
| <b>• Secreted Proteins/Agonists from α Granules and Dense Granules</b>           |
| Ligands (fibrinogen, fibronectin, thrombospondin, vitronectin, vWF)              |
| Enzymes (α <sub>2</sub> -antiplasmin, Factors V, VIII, and XI)                   |
| Antiheparin (platelet factor 4)                                                  |
| ADP, serotonin                                                                   |
| <b>• Secreted Cytosolic Factor XIII</b>                                          |

GP = glycoprotein; vWF = von Willebrand factor; ADP = adenosine diphosphate.

velocities of the arterial circulation is shown in Fig 10-4, where parallel planes of blood are moving at different velocities,<sup>166</sup> which create shear stress. Shear rates at the vessel wall vary between 500sec<sup>-1</sup> in larger arteries to 5000sec<sup>-1</sup> in the smallest arterioles and can reach levels of 3000 to 10,000sec<sup>-1</sup> (and even higher) at the surface of atherosclerotic plaques with stenosis.

One of the forces enhancing platelet adhesion to the vessel wall in the arterial circulation is radial dispersion, the tendency of larger cells to stream in the center of the vessel where shear is lowest; this phenomenon effectively pushes the smaller platelets toward the vessel wall. This size-dependent flow may explain how the correction of severe anemia can slow or stop bleeding.<sup>167</sup> This effect also underscores the importance of platelets in arterial hemostasis; reductions in platelet number or function may be associated with catastrophic arterial hemorrhage. By contrast,

the lesser shear forces experienced in the venous circulation (20 to 200sec<sup>-1</sup>) permit more random cell movement and greater time for localized coagulation reactions to occur, making fewer demands for adequate platelet number and function.

Given the velocity of blood flow in an artery, platelets must rapidly activate and adhere to the injured vessel. Two molecules in the subendothelium are critical for this: vWF<sup>168</sup> and collagen. Control of bleeding in vessels under the highest shear stresses is dependent on adequate vWF concentration and function.<sup>169</sup> The largest multimeric forms of vWF, which are immobilized by adherence to exposed subendothelial collagen, bind platelet GPIb<sup>170</sup> in response to high shear stress (Fig 10-4). This is a rapid but low-affinity event that markedly slows the circulating platelets but does not firmly adhere them to vWF. With platelets now tumbling over the subendothelium, transmembrane signaling is produced by both high shear stress and the GPIb-vWF interaction<sup>171</sup>; this results in platelet shape change and a conformationally activated GPIIb/IIIa, which can bind either to fibrinogen or larger vWF multimers. The adhesion of GPIIb/IIIa-vWF is a higher affinity interaction than the GPIb-vWF bond and adheres platelets firmly into the subendothelial vWF. Once a layer of platelets is adherent, vWF bound to GPIb on the uppermost adherent platelets serves to recruit additional platelets from the vascular flow into the growing platelet plug.

At more moderate shear stress, GPIb-vWF adhesion is supplemented by subendothelial collagen binding itself to platelet GPIa/IIa (Table 10-1).<sup>172</sup> Thus, subendothelial vWF and collagen act cooperatively to initiate platelet adhesion, with the former predominating at higher shear rates. Collagen also directly activates platelets by binding to platelet GPVI at a second locus.<sup>173</sup> The congenital absence of any of these critical platelet adhesion receptors—GPIIb/IIIa, GPIb,<sup>52</sup> GPVI,<sup>174</sup> or GPIa/IIa<sup>175</sup>—results in a significant hemostatic defect, correctable only by platelet transfusion (see congenital platelet disorders below). Similarly, decreases in the concentration of vWF, especially the larger multimeric forms, or abnormal vWF function predispose to bleeding.<sup>176</sup>

### Platelet Activation

Adherent platelets undergo a series of interdependent processes collectively referred to as activation with five major sequelae: 1)



**Table 10-2.** Drugs that Inhibit Platelet Function

- ADP receptor antagonists (clopidogrel, prasugrel, ticlopidine)
- Antibiotics (cephalosporins, nitrofurantoin, penicillins)
- Anti-GPIIb/IIIa or anti-RGD compounds (abciximab, eptifibatide, tirofiban)
- Cardiac drugs (nitroglycerin, nitroprusside)
- Cyclooxygenase inhibitors (aspirin, nonsteroidal antiinflammatory drugs that are COX-1/2 nonselective)
- Heparin (unfractionated and low-molecular-weight)
- Miscellaneous (dextran, hydroxyethyl starch, garlic, ginkgo biloba)
- Phosphodiesterase inhibitors (cilostazol, dipyridamole)

ADP = adenosine diphosphate; GP = glycoprotein.

release of adhesive ligands that stabilize platelet-platelet binding, 2) recruitment of additional platelets from flowing blood, 3) vasoconstriction to slow bleeding, 4) localization and acceleration of platelet-associated fibrin formation, and 5) clot protection from fibrinolysis.

The basis of the platelet plug is a platelet-ligand-platelet matrix; fibrinogen and vWF serve as bridging ligands, and both are stored within platelet  $\alpha$ -granules. The  $\alpha$ -granules fuse with the open canalicular system following platelet activation, releasing both fibrinogen and vWF; GPIIb/IIIa binds to the fibrinogen molecule and to the larger vWF multimers via RGD sites, creating the platelet-ligand-platelet matrix.<sup>177</sup> GPIIb/IIIa is the most abundant glycoprotein on the platelet surface, with approximately 50,000 copies on the resting platelet; additional GPIIb/IIIa receptors within the cytosol are mobilized to the surface after activation. The importance of these platelet adhesive mechanisms is underscored by the potent antiplatelet drugs that target GPIIb/IIIa to block arterial clotting (Table 10-2).

Platelets are continuously recruited into the platelet plug by the actions of local agonists (collagen, epinephrine, and thrombin) and by localized release of additional agonists (ADP, serotonin) from adherent platelets. Both collagen (as noted above) and thrombin interact with specific platelet receptors to strongly activate platelets. Although epinephrine by itself is not a powerful platelet agonist, stimulation of the  $\alpha$ -adrenergic receptor on platelets primes them for synergistic activation to other agonists, including ADP. Another activating compound released directly from activated platelets is thromboxane  $A_2$ , which is formed in the platelet cytosol after cyclooxygenase cleavage of arachidonic acid and then released into the clot milieu.<sup>178</sup> Thromboxane  $A_2$  is both a platelet agonist and a vasoconstrictor, and it is rapidly degraded to its inert by-product, thromboxane  $B_2$ . Cyclooxygenase activity is *irreversibly* inhibited by aspirin, thereby blocking thromboxane  $A_2$  formation for the lifetime of that platelet, unlike nonsteroidal anti-inflammatory drugs (NSAIDs), which *reversibly* inhibit cyclooxygenase activity.

Other platelet agonists are liberated into the clot microenvironment by fusion of platelet dense granules and  $\alpha$ -granules with the platelet external membrane, producing extrusion of granule contents (Table 10-1) including fibrinogen, fibronectin, vWF, and thrombospondin. Translocation to the platelet membrane by

$\alpha$ -granules also results in external membrane expression of P-selectin, which contributes directly to platelet-heterotypic cell adhesion.<sup>179</sup> Adherence of activated platelets via P-selectin to neutrophils and monocytes in the blood has been demonstrated to be a marker for platelet activation in several clinical circumstances, including cardiopulmonary bypass,<sup>180</sup> thrombotic thrombocytopenic purpura,<sup>181</sup> and unstable coronary syndromes.<sup>182</sup> This P-selectin-mediated adhesive interaction may serve to target heterotypic cell populations to areas of inflammation or thrombosis,<sup>183</sup> upregulate procoagulant (tissue factor)<sup>184</sup> and/or inflammatory (TNF $\alpha$ ) activity,<sup>185</sup> and promote transcellular metabolic cooperation.<sup>186</sup>

Platelet dense granules contain serotonin which, like thromboxane  $A_2$ , is both a platelet agonist and a vasoconstrictor.<sup>187</sup> Another dense-granule constituent, ADP, acts purely as a platelet agonist without vasoactive properties (Table 10-1). The importance of thromboxane  $A_2$ - and serotonin-induced vasoconstriction is not entirely clear. However, by decreasing the vessel diameter, vasoconstriction may increase shear stress and thereby facilitate additional recruitment of platelets to the clot. The importance of dense-granule release to the maintenance of hemostasis is manifested by the severe hemorrhage noted in patients with congenital dense-granule deficiencies (eg, Hermansky-Pudlak syndrome and storage pool disease). Therefore, platelet activation serves to amplify platelet adhesion and optimize the platelet surface for fibrin-generating procoagulant activity by interaction of platelets with the soluble coagulation cascade.

### Platelet Interaction with Soluble Coagulation Factors

Tissue injury and low levels of circulating activated clotting factors physiologically initiate the clotting cascade, which is then propagated by enzyme complexes that efficiently assemble on the phospholipid membrane surface supplied by platelets. Tissue factor (TF) expressed after endothelial injury binds to circulating Factor VIIa, and the Factor VIIa-TF complex subsequently binds to its zymogen substrates, Factors IX and X, on the activated platelet membrane to form the extrinsic “tenase” complex.<sup>188</sup> Platelet activation increases external membrane expression of the negatively charged, procoagulant phosphatidylserine (PS) moiety, which can be detected either by calcium-dependent binding to annexin V or by procoagulant assays, the latter because PS on the activated platelet surface enhances both the tenase and prothrombinase reactions. Activated platelets also provide surface receptors for Factors Xa, IXa, and Va; Factor V is also secreted from the platelet  $\alpha$ -granule, and platelet-derived Factor V appears to significantly contribute to overall Factor V function, as noted in Factor V-deficient patients after platelet transfusion.<sup>188</sup> Platelet receptor binding simultaneously protects activated factors from circulating inhibitors and orients factors on the plasma membrane such that they interact closely with PS to accelerate thrombin generation.<sup>189</sup>

Physiologic cell-based hemostasis can therefore be modeled as: TF-VIIa converts Factor X to Xa and Factor IX to IXa, both



of which are bound to platelet receptors. The intrinsic tenase complex on the platelet membrane is formed initially by TF-induced Factor IXa formation; Factor IXa binds to its cofactor, Factor VIIIa, and its zymogen substrate Factor X to amplify production of Factor Xa on the platelet surface (Fig 10-5). The platelet Factor Xa receptor is closely associated with platelet-bound Factor Va; together with free  $\text{Ca}^{2+}$  and platelet membrane PS, these factors bind prothrombin (Factor II) to form the prothrombinase complex,<sup>190</sup> thereby generating thrombin, albeit in relatively small amounts. This initially formed thrombin feeds back to activate Factor VIII to VIIIa (which binds to platelet-bound Factor IXa), Factor V to Va (which binds to its platelet receptor), and Factor XI to XIa. Once these initial amounts of thrombin activate more platelets and generate appreciable quantities of Factor XIa<sup>191</sup> and cofactors Factor Va and VIIIa on the platelet surface,<sup>192</sup> activation of the more kinetically favorable intrinsic pathway occurs to amplify the clotting cascade, thereby increasing the rate of Factor Xa and thrombin generation exponentially.<sup>193</sup> This rapid thrombin generation cleaves fibrinogen to fibrin monomers, which combine to form a fibrin matrix integrated with adherent platelets. Factor XIIIa, a transamidase produced by the action of thrombin on either plasma or platelet-released Factor XIII, converts the soluble fibrin clot into an insoluble fibrin polymer and effectively incorporates  $\alpha_2$ -antiplasmin into the clot to inhibit plasmin-mediated dissolution.<sup>194</sup> Finally, the platelet plug undergoes clot retraction, which additionally protects the platelet-fibrin matrices from lysis by plasmin.<sup>195</sup>

### Acquired Qualitative Platelet Defects

The normal ability of platelets to adhere to damaged vessels and recruit additional platelets into the clot is critical for primary hemostasis, especially when patients are hemostatically challenged by trauma or surgery. Drugs are the most common cause of acquired platelet dysfunction. Aspirin irreversibly acetylates platelet cyclooxygenase-1, blocking activation via the arachidonic acid pathway; all exposed platelets are irreversibly affected even after aspirin is discontinued. By contrast, NSAIDs *reversibly* inhibit cyclooxygenase-1 such that platelet function is restored within 24 to 48 hours after discontinuation of the drug. However, surgical bleeding is often unaffected in the setting of either concomitant aspirin or NSAID therapy,<sup>196</sup> and aspirin is often continued through surgery in patients who are at risk for stroke or myocardial infarction. However, excessive surgical bleeding caused by aspirin-induced platelet dysfunction can be reduced by treatment with desmopressin acetate (DDAVP).<sup>197</sup> When DDAVP is ineffective, platelet transfusion consisting of 4 to 6 whole-blood-derived units or a single apheresis unit will restore primary hemostasis in aspirin-treated patients. Antiplatelet agents are also commonly used in combination, and beyond inhibiting the aggregation response, these drugs can also block platelet procoagulant function<sup>198</sup>; hence, platelet transfusion should be a consideration in any such patient facing major surgery or experiencing significant bleeding. Platelet dysfunction and bleeding caused by drugs other than aspirin or NSAIDs (Table 10-2) can

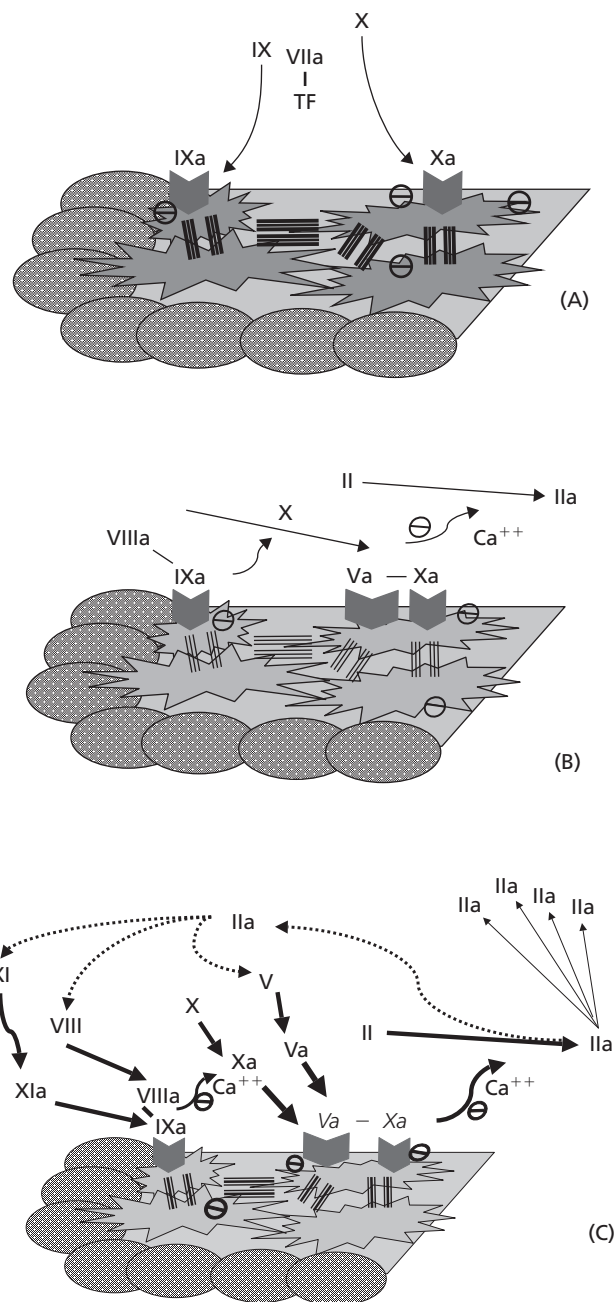


**Figure 10-5.** Platelet-based hemostasis. (A) Activated Factor VII – tissue factor (TF) complex converts Factor X to Xa and Factor IX to IXa, which are bound to platelet receptors. (B) Factor IXa binds to its cofactor, Factor VIIIa, and its zymogen substrate Factor X to form the tenase complex, creating Factor Xa on the platelet surface. The platelet Factor Xa receptor is closely associated with platelet-bound Factor Va; together with free  $\text{Ca}^{2+}$  and platelet membrane phosphatidylserine ( $\Theta$ ), these factors bind prothrombin (Factor II) to form the prothrombinase complex, thereby generating small amounts of thrombin (Factor IIa). (C) Thrombin feeds back to activate platelets and factors: Factor VIII to VIIIa (which binds platelet-bound Factor IXa), Factor V to Va (bound to its platelet receptor), and Factor XI to XIa (which forms more Factor IXa). Activation of the more kinetically favorable intrinsic pathway now occurs to exponentially increase the rate of Factor Xa and Factor IIa generation.

be similarly treated.<sup>199</sup> Proteins that accumulate in renal failure effectively poison platelet adhesive function.<sup>200</sup> Adequate dialysis and hematocrit maintenance preserve platelet function, but bleeding with acute renal failure is seen. Platelet transfusion may be used in life-threatening uremic bleeding, but transfused platelets rapidly acquire the uremic defect; therefore, acute uremic platelet dysfunction is generally treated with DDAVP or cryoprecipitate.<sup>201</sup> Long-term estrogen therapy has been shown to be efficacious in renal failure patients prone to chronic hemorrhage.

### Congenital Platelet Dysfunction

Inherited qualitative platelet defects include abnormalities of platelet receptors, platelet procoagulant activity, and granules. Two relatively rare, but well-characterized, platelet receptor disorders are Bernard-Soulier syndrome (BSS)<sup>202</sup> and Glanzmann thrombasthenia (GT).<sup>203</sup> BSS affects vWF binding to platelets via decreased surface expression of platelet GPIb (the primary vWF receptor) or diminished GPIb function. BSS is characterized by a mild-to-moderate bleeding disorder with mild thrombocytopenia, increased bleeding time, and large platelets; the diagnosis is occasionally not made until adulthood. Laboratory testing shows an absent platelet aggregation response to ristocetin (which agglutinates platelets through GPIb-vWF interaction) but with normal plasma vWF antigen and normal ristocetin cofactor activity. GT is characterized by bleeding in childhood with an increased bleeding time and abnormally low levels of platelet GPIIb/IIIa expression (a receptor for both vWF and fibrinogen) or normal expression but absent GPIIb/IIIa function. Platelet aggregation testing in GT shows absent or diminished response to all agonists (ADP, epinephrine, collagen, arachidonic acid) except ristocetin. Platelet transfusions are effective for bleeding symptoms in both BSS and GT. However, because of the high risk of alloimmunization with frequent platelet transfusions,<sup>204</sup> transfusion is usually reserved for life-threatening bleeding, and most patients are successfully managed with conservative therapy.

Inherited platelet granule disorders are defined by the type of granule that is absent or defective.<sup>205</sup> Storage pool disease is characterized by a relative decrease or absence of dense granules, resulting in symptoms of moderate-to-severe mucosal bleeding. Defective release of dense-granule constituents impairs the ability of platelets to recruit and activate other platelets in the microenvironment. The laboratory diagnosis of storage pool disease is characterized by diminished or absent secondary wave aggregation to weak platelet agonists such as epinephrine and ADP.<sup>206</sup> Hermansky-Pudlak syndrome is one form of dense-granule deficiency and is accompanied by oculocutaneous albinism and mild thrombocytopenia. Patients with Hermansky-Pudlak syndrome demonstrate excessive bleeding with trauma or surgery and occasionally have spontaneous bleeding. Chediak-Higashi disease is a general granule disorder characterized by mild bleeding, partial albinism, and recurrent pyogenic infections.

The absence of platelet  $\alpha$ -granules and/or their contents is gray platelet syndrome, so-called because platelets seen on the peripheral smear lack the normal basophilic staining.<sup>207</sup> Patients with gray platelet syndrome have a mild bleeding history, and

there is diminished platelet aggregation to epinephrine, ADP, and collagen. Patients with platelet granule disorders can be successfully treated conservatively by avoidance of aspirin and other antiplatelet drugs (Table 10-2), and hormonal control of menses. However, when significant bleeding occurs, platelet transfusions are necessary to correct hemostasis.

Patients with bleeding caused by the extremely rare Scott syndrome<sup>208</sup> have normal platelet aggregation and secretion,<sup>209</sup> but their platelets are unable to translocate anionic phospholipids from the internal to the external platelet membrane with activation.<sup>210</sup> These platelets also showed impaired ability to generate platelet microparticles, as well as a decrease in the number and function of inducible Factor Va receptors on the platelet surface. Thus, platelets in Scott syndrome do not provide a membrane surface that optimally provides sites for assembly of the intrinsic tenase and prothrombinase complexes.<sup>211</sup> As with another rare disorder of platelet procoagulant function (Factor V Quebec, a platelet defect in which most proteins of the  $\alpha$ -granules are probably degraded),<sup>212,213</sup> platelet transfusion is effective for clinically significant bleeding.

### Platelet Function and Survival After Transfusion

Preparation and storage of platelets for transfusion results in partial platelet activation and some loss of metabolic function. Stored platelets gradually lose their *de novo* ability to aggregate, and platelet-matrix adhesion capabilities are similarly compromised as measured by *in-vitro* assays.<sup>214-216</sup> Moreover, the longer platelets are stored, the shorter their subsequent survival in animal models<sup>217</sup>; however, in humans, the clinical status of the transfusion recipient also influences the recovery and survival of transfused platelets.<sup>218</sup> Despite the functional and survival lesions of platelet storage, transfused platelets will appear in the circulation and restore hemostasis in patients bleeding from thrombocytopenia or platelet dysfunction.

Platelet transfusion in humans with chemotherapy-induced thrombocytopenia results in a rapid increase in whole blood platelet aggregation and adenosine triphosphate release.<sup>215,219</sup> One reason for stored platelet recovery of function after transfusion is the restored milieu of normal pH and metabolic substrates for the transfused platelet.<sup>220</sup> This restoration of platelet function is especially convincing for platelet procoagulant abilities based on phospholipid expression to agonists. With respect to circulating transfused platelets, modeling data suggest that stored platelets can survive after transfusion in humans for an average of 6 days,<sup>221</sup> and these findings have been confirmed by *in-vivo* studies.<sup>222,223</sup> Based on these encouraging results of stored platelet function and survival, research<sup>224,225</sup> has been focused on finding methods to preserve stored platelets for longer periods without compromising function or increasing the risk of bacterial contamination.

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## Summary

In conclusion, recently acquired knowledge of the molecular mechanisms that regulate megakaryocytopoiesis and platelet

production—together with new developments in the generation and availability of various thrombopoietic agents—provide a strong basis for future therapy of thrombocytopenic states. These thrombocytopenic pathologies can be qualified according to decreased production or increased clearance by the enumeration of circulating reticulated platelets. Pharmacologic thrombopoietin moieties have demonstrated promise at restoring platelet counts in both immunologic and hypoproliferative thrombocytopenias, especially for the latter when there is residual megakaryocyte mass. New understanding of platelet hemostatic mechanisms has led to multiple strategies for blocking pathologic platelet-dependent thromboses, as well as an improved ability to treat bleeding caused by platelet dysfunction and thrombocytopenia. Future investigations will likely result in improved thrombopoietin therapies to reverse low platelet counts and improved storage conditions for platelets in order to provide maximum hemostatic benefit for platelet transfusion recipients.

## Disclaimer

The authors have disclosed no conflicts of interest.

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Platelets express a variety of immunogenic markers on the cell surface. Some of these antigens are shared with other cell types as in the case of HLA antigens, which are shared with virtually all nucleated cells in the body, whereas others are observed to be essentially platelet specific. This chapter reviews the antigens expressed on platelets, the various patterns of alloimmunization to these antigens, and their impact on platelet transfusion responses. Strategies to treat and prevent alloimmunization are summarized.

## Antigens on the Platelet Surface

### Antigens Shared with Other Tissues

#### HLA Antigens

HLA Class I antigens are the principal antigens shared by platelets and lymphocytes. Indeed, platelets are the major source of HLA Class I antigens in whole blood.

Although platelets ultimately derive from nucleated precursors (the megakaryocytes) and hence might be anticipated to acquire their HLA antigens through mechanisms in common with other nucleated cells, consensus as to the source of HLA Class I structures in circulating, mature platelets has developed only recently. Early studies<sup>1,2</sup> demonstrated that, in vitro, platelets do absorb and express soluble HLA antigens after incubation in plasma. Other studies showing that HLA Class I molecules can apparently be eluted from platelets by incubation in low pH chloroquine or citrate solutions<sup>3,4</sup> suggested that these antigens were not firmly embedded in the membrane and were likely absorbed from the surrounding plasma. These studies suggested that HLA absorbed from plasma contributed significantly to the total HLA Class I present on platelets. However, only small amounts of HLA were absorbed by platelets in these studies, and

it is now recognized that low pH treatment of platelets does not “strip” the HLA Class I heavy chain from the cell membrane of platelets. Rather, acid treatment of platelets actually disrupts the trimolecular complex of HLA Class I heavy chain, peptide, and  $\beta_2$ -microglobulin; destroying antigenic epitopes and preventing the binding of specific HLA antibodies.<sup>5,6</sup>

It is now clear that most HLA Class I molecules on platelets are integral membrane proteins persisting from the megakaryocyte stage of development. Evidence for this includes studies of phorbol ester stimulation of platelets that resulted in phosphorylation of their HLA molecules,<sup>7</sup> and mRNA from platelets that is capable of producing small amounts of HLA Class I<sup>8</sup>—both of which are consistent with HLA molecules being an integral part of the platelet membrane. In addition, peptides presented by HLA-A2 molecules on human platelets have been isolated and sequenced, and shown to be identical to those commonly expressed by HLA-A2 on nucleated cells.<sup>6</sup> One ubiquitously expressed peptide is derived from the megakaryocyte-platelet-specific glycoprotein (GP) IX. Detectable HLA Class I surface molecules that were removed by treatment with pH 3.0 citrate could be restored only after addition of exogenous  $\beta_2$ -microglobulin and peptide ligand. This indicates that platelets themselves are not able to load HLA molecules with endogenous peptides, but that this occurs during HLA protein synthesis at the megakaryocyte stage.<sup>6</sup> It is clear that the vast majority of HLA Class I molecules on platelets are intrinsic transmembrane proteins synthesized and acquired at the megakaryocyte stage before platelets become cytoplasmic fragments.

Another controversy centers around the number of HLA Class I molecules expressed on the platelet surface. Estimates vary from approximately 15,000 to 120,000.<sup>9,10</sup> Using a direct assay with a <sup>125</sup>I-labeled monoclonal antibody specific for the Fc portion of human immunoglobulin G (IgG), the number of anti-HLA IgG molecules binding to specific HLA antigens at saturation ranged from 870 to 11,000 per platelet per antigen, depending on the antigen.<sup>10</sup> The same number of HLA Class I antigens on platelets were found in a study of density-fractionated platelets.<sup>11</sup> A quantitative competitive binding assay for platelet-associated HLA

found a much higher number of molecules per platelet (up to 120,000).<sup>12</sup> A mean of 81,587 molecules per platelet was found in 12 healthy individuals.

Several studies attempting to quantitate HLA on platelets demonstrated variable expression of specific antigens in different individuals.<sup>9,12,13</sup> The co-variation of the quantity of Bw4/6 antigens with specific private HLA-B antigens and the variability of B44 expression have been demonstrated repeatedly.<sup>9,13</sup> Platelets from the same individual also seem to differ in HLA antigen expression in that lower-density platelet cohorts were found to have 42% more HLA-A2 molecules than high-density cohorts.<sup>11</sup> More variability in A2, B35, and B62 antigen expression on platelets was measured among different individuals. Here again, the levels of specific antigens varied three- to fivefold among individuals and also on the platelets of the same individual.<sup>12</sup>

Only HLA Class I molecules appear to be present on the platelet membrane, and of the Class I loci, only the A and B antigens appear to be significant causes of alloimmunization in transfusion recipients. HLA-C antigens are also present and reported to be expressed at approximately the same density as A antigens;<sup>14</sup> however, transfused patients are rarely sensitized to HLA-C markers.<sup>15</sup> HLA Class II molecules are not generally found on platelet membranes. An apparent exception is a report of their detection on platelets of a patient with acute autoimmune thrombocytopenia.<sup>16</sup> Although Class II antigens have been detected on megakaryoblasts from patients with myeloproliferative diseases and in megakaryoblast colony-forming unit colonies in cultures of normal marrow, this was the first report of Class II antigen expression on circulating platelets. The implications of this finding in the pathogenesis of autoimmunity are intriguing.

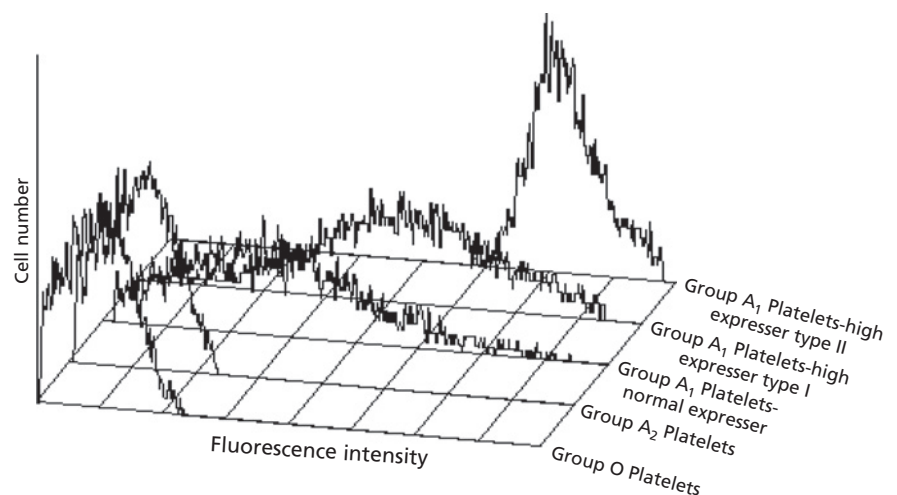
### ABH Blood Group Antigens

The ABH blood group system is another important non-platelet-specific antigen system on platelets. Similar to HLA antigens, the amount of ABH substance on platelets varies not only from person to person but also among platelets in the same person. The variable distribution of group A substance has been

demonstrated on platelets using flow cytometry.<sup>17</sup> This distribution of ABH could explain why, in some platelet transfusions, there is a rapid destruction of a subset of ABO-incompatible cells followed by near-normal survival of the remaining cells.<sup>18</sup> Greater recovery was seen for group B platelets than for group A platelets, attributable perhaps to the lower levels of B antigens on donor platelets<sup>19,20</sup> and the lower levels of B-specific isoagglutinins in recipient plasma.<sup>18</sup> The number of B sites on platelets is considered to be about half the number reported for A. The number of A antigens per platelet estimated using an IgG murine monoclonal antibody specific for A substance and immunofluorescence flow cytometry showed a wide range (2,100 to 16,000 molecules/platelet) and was donor dependent.<sup>19</sup> Individuals of the subgroup A<sub>2</sub> have no detectable A antigens on their platelets<sup>19-22</sup> (Fig 11-1), and A<sub>2</sub> platelets can be substituted successfully for group O, even for transfusion to refractory group O patients with high-titer anti-A,B.<sup>22</sup>

For many years it was thought that most ABH antigens on platelets (as for HLA antigens) were absorbed from the plasma. Group O platelets incubated in group A or group B plasma could be shown to acquire soluble A or B substance detectable with anti-A or anti-B typing reagents in agglutination tests.<sup>23,24</sup> More recent studies show that a great majority of blood group A, B, and H antigens on platelets are expressed on many of the integral platelet membrane glycoproteins including GPIIb, GPIIIa, GPIV, GPV, PECAM-1, GPIb/IX, GPIa/IIa, and CD109<sup>20,21,25-28</sup> with GPIa/IIa expressing the most ABH substance per GP molecule and GPIIb and PECAM-1 expressing the majority of ABH per platelet.<sup>19,21</sup> A- and B-UDP sugars are attached in the golgi of the megakaryocyte to the Type II H precursor chains present on these platelet glycoproteins. Studies by several groups using monoclonal probes specific for Type II H chains have demonstrated that this membrane-intrinsic form of H substance is present on platelets and detectable in varying quantities according to the ABO group of the platelet donor [greatest in group O and A<sub>2</sub> platelets; less in groups B, A<sub>1</sub>, and A<sub>1</sub>B; and least in O<sub>H</sub> (Bombay)].<sup>19,20,24,25</sup> Although it is likely that a small, but significant, amount of ABH on platelets is acquired by absorption of glycolipids

**Figure 11-1.** Fluorescence histograms of platelets after incubation with a fluorescent-labeled monoclonal antibody specific for the blood group A antigen and immunofluorescence detection by flow cytometry. Group A<sub>2</sub> platelets are not distinguishable from group O platelets—no A antigens detected. Normal group A<sub>1</sub> platelets (middle histogram) show great variation in A antigen expression—broad histogram. Platelets from a Type 1 high expresser, like the normal expresser, give a broad histogram, but have overall higher levels (histogram shifted to the right) of A antigen. Type II high expressers have much higher levels and show a more homogeneous (narrow histogram at top) expression of A antigens per platelet.



expressing these antigens from the surrounding plasma, the majority, as for HLA, is intrinsically derived at the megakaryocyte stage.

One report from Japan described a group O patient who failed to respond to two of 12 ABO-incompatible HLA-matched platelet transfusions.<sup>20</sup> Further evaluation determined that the platelets in the two unsuccessful transfusions were from donors who expressed unusually high amounts of group B substance on their platelets, up to 20 times that found on group B, HLA-matched platelets that were successfully transfused in this patient. Follow-up investigation of 313 healthy Japanese blood donors revealed that approximately 7% had platelets that carried significantly more A or B antigen than the mean level of these antigens on platelets determined for the entire group. This phenotype, designated “high expresser,” was found to be inherited as a dominant trait and was unrelated to secretor phenotype, but did correlate with elevated serum glycosyltransferase activity.<sup>20</sup> These findings were confirmed in studies of 200 blood donors of European ancestry that further defined two subgroups of high expressers of platelet A<sub>1</sub> antigens, termed Type I and Type II.<sup>19</sup> Type I high expressers had elevated platelet A<sub>1</sub> antigen levels (about three times the normal expression), elevated serum A<sup>1</sup>-glycosyltransferase activity, markedly decreased H levels, and a heterogeneous distribution of A<sub>1</sub> antigens on individual platelets (Fig 11-1). Type II high expressers were characterized as having platelet A<sub>1</sub> antigen levels that averaged seven times the normal expression, higher A<sup>1</sup>-transferase activity than even Type I individuals, a homogeneous distribution of platelet A<sub>1</sub> antigens (Fig 11-1), and the majority of the antigens were found to be expressed on platelet GPIIb and PECAM-1.<sup>19</sup> This marked interdonor variability in A and B substance expression on platelets may offer an explanation for the nonuniform refractory response some patients develop to ABO-incompatible platelet transfusions (see below). The high expression phenomenon might also explain some cases of fetal and neonatal alloimmune thrombocytopenia (FNAIT). Curtis et al<sup>29</sup> described two group B infants with moderately severe thrombocytopenia at birth who inherited the Type II high expresser phenotype from their A<sub>2</sub>B father. They were born to a group O mother and the thrombocytopenia was apparently caused by transplacentally acquired, high-titer maternal IgG antibodies specific for blood group B. Antibodies against platelet-specific antigens could not be detected in the mother’s serum, and she later gave birth to a third infant who inherited the A<sub>2</sub> blood group instead of group B and was born with a normal platelet count. Two unresolved cases of suspected FNAIT, in which the fathers were both Type II high expressers of platelet A antigens, have also been reported.<sup>19</sup> Maternal/fetal ABO incompatibility should, therefore, be considered as a possible cause of FNAIT when antibodies reactive with platelet-specific antigens cannot be demonstrated.

### Other Antigens on Platelets

Other red cell antigens have been sought on the platelet surface. Ii, Lewis, and P antigens were demonstrated by using flow cytometry,<sup>26</sup> but Rh, Duffy, Kidd, Kell, and Lutheran-b were not detected by a two-stage radioimmunometric assay.<sup>30</sup>

Platelet GP IV or CD36 is expressed on various human cells including platelets, monocytes/macrophages, capillary endothelium, erythroblasts, and adipocytes.<sup>31,32</sup> Some apparently normal individuals lack CD36 on their platelets (Type II deficiency) or platelets and monocytes (Type I deficiency).<sup>33</sup> CD36 deficiency is common in Asian populations (3-11%)<sup>34</sup> and African populations (3-6%),<sup>35,36</sup> but is extremely rare in European populations (0.1%).<sup>35,37</sup> It is interesting that the most common gene mutations responsible for CD36 deficiency in those of Asian ethnicity and African ethnicity are different—C→T<sub>478</sub> point mutation in exon 4 (Asians<sup>33,38</sup>) and T→G<sub>1264</sub> stop mutation in exon 10 (Africans<sup>39</sup>). The higher frequency of CD36 deficiency in the former groups is thought to be related to their living in regions of the world where malaria is endemic. CD36 is a known receptor for red cells infected with *Plasmodium falciparum*; thus, it is believed that CD36 deficiency may afford partial resistance to malaria infection. However, one report suggests CD36 deficiency may actually be a risk factor for more severe forms of malaria infection.<sup>39</sup> Type I CD36-deficient people can become immunized from transfusion or pregnancy and make antibodies against CD36 that have been implicated in cases of multiple platelet transfusion refractoriness,<sup>34,40-42</sup> posttransfusion purpura (PTP),<sup>43</sup> and FNAIT.<sup>37,44</sup> Transfusion of CD36-deficient platelets has been reported to be successful in supporting patients immunized against CD36.<sup>37,41,42,44</sup>

CD109 is a 175-kD glycoprotein found on activated T cells, cultured endothelial cells, several tumor cell lines, and platelets.<sup>45</sup> Two alloantigens named Gov<sup>a</sup> (HPA-15b) and Gov<sup>b</sup> (HPA-15a), were found on platelet CD109,<sup>46</sup> and unlike most platelet-specific alloantigens, both alleles are highly expressed: 77% (HPA-15a) and 65% (HPA-15b) in those of European ethnicity (Table 11-1). Recent cloning and sequencing of the CD109 gene showed that it is a member of the α<sub>2</sub>-macroglobulin/complement gene family,<sup>47</sup> and a single nucleotide polymorphism A→C<sub>2108</sub> resulting in a Tyr:Ser<sub>703</sub> change in the protein defines the HPA-15a/b alloantigens.<sup>48</sup> The number of HPA-15 antigens expressed on platelets is small (<2000 molecules/platelet), and the antigens are labile in storage, making detection of HPA-15 antibodies difficult with the use of currently available serologic assays. These properties of HPA-15 would suggest that alloimmunization to these antigens may be underreported. Results from several studies using optimal assay conditions show that antibodies to HPA-15a or HPA-15b are uncommon (0.2-1.0%) in sera collected from women having given birth to infants affected with FNAIT, but more frequent in sera from patients with PTP (2%) and platelet transfusion refractoriness (3-4%).<sup>49-51</sup> These data suggest that HPA-15 may be as immunogenic as the HPA-5 antigen system, which is second only to the highly immunogenic HPA-1a (PI<sup>A1</sup>) platelet antigen system.

### Platelet-Specific Antigens

Antibodies recognizing platelet-specific antigens have been discovered in three clinical situations: mothers who give birth to infants with FNAIT; patients who develop dramatic thrombocytopenia

**Table 11-1.** Human Platelet Antigens

| Antigens                    | Other Names          | Phenotypic Frequency*                       | Glycoprotein Location/Amino Acid Change    | Nucleotide Substitution                   |
|-----------------------------|----------------------|---------------------------------------------|--------------------------------------------|-------------------------------------------|
| HPA-1a (Pl <sup>A1</sup> )  | Pl <sup>A</sup> , Zw | 72% a/a                                     | GP1IIa /                                   | T→C <sub>196</sub>                        |
| HPA-1b (Pl <sup>A2</sup> )  |                      | 26% a/b<br>2% b/b                           | Leu↔Pro <sub>33</sub>                      |                                           |
| HPA-2a (Ko <sup>b</sup> )   | Ko, Sib              | 85% a/a                                     | GP1Ib /                                    | C→T <sub>524</sub>                        |
| HPA-2b (Ko <sup>a</sup> )   |                      | 14% a/b<br>1% b/b                           | Thr↔Met <sub>145</sub>                     |                                           |
| HPA-3a (Bak <sup>a</sup> )  | Bak, Lek             | 37% a/a                                     | GP1Ib /                                    | T→G <sub>622</sub>                        |
| HPA-3b (Bak <sup>b</sup> )  |                      | 48% a/b<br>15% b/b                          | Ile:Ser <sub>843</sub>                     |                                           |
| HPA-4a (Pen <sup>a</sup> )  | Pen, Yuk             | >99.9% a/a                                  | GP1IIa /                                   | G→A <sub>526</sub>                        |
| HPA-4b (Pen <sup>b</sup> )  |                      | <0.1% a/b<br><0.1% b/b                      | Arg:Gln <sub>143</sub>                     |                                           |
| HPA-5a (Br <sup>b</sup> )   | Br, Hc, Zav          | 80% a/a                                     | GP1a /                                     | G→A <sub>648</sub>                        |
| HPA-5b (Br <sup>a</sup> )   |                      | 19% a/b<br>1% b/b                           | Glu:Lys <sub>505</sub>                     |                                           |
| HPA-6bw                     | Ca <sup>a</sup> , Tu | <1% b/b                                     | GP1IIa / Arg↔Gln <sub>489</sub>            | A→G <sub>1564</sub>                       |
| HPA-7bw                     | Mo <sup>b</sup>      | <1% b/b                                     | GP1IIa / Pro↔Ala <sub>407</sub>            | G→C <sub>1317</sub>                       |
| HPA-8bw                     | Sr <sup>a</sup>      | <0.1% b/b                                   | GP1IIa / Arg↔Cys <sub>636</sub>            | T→C <sub>2004</sub>                       |
| HPA-9bw                     | Max <sup>a</sup>     | <1% b/b                                     | GP1Ib / Val:Met <sub>837</sub>             | A→G <sub>2603</sub>                       |
| HPA-10bw                    | La <sup>a</sup>      | 1% b/b                                      | GP1IIa / Arg:Gln <sub>62</sub>             | A→G <sub>281</sub>                        |
| HPA-11bw                    | Gro <sup>a</sup>     | <0.5% b/b                                   | GP1IIa / Arg:His <sub>633</sub>            | A→G <sub>1996</sub>                       |
| HPA-12bw                    | ly <sup>a</sup>      | 1% b/b                                      | GP1Ib <sub>3</sub> / Gly:Glu <sub>15</sub> | A→G <sub>141</sub>                        |
| HPA-13bw                    | Sit <sup>a</sup>     | <1% b/b                                     | GP1a / Met:Thr <sub>799</sub>              | T→C <sub>2531</sub>                       |
| HPA-14bw                    | Oe <sup>a</sup>      | 1% b/b                                      | GP1IIa / Del:Lys <sub>611</sub>            | A→G <sub>1929-31</sub>                    |
| HPA-15a (Gov <sup>b</sup> ) | a/a                  | 35%                                         | CD109 / Tyr:Ser <sub>703</sub>             | A→C <sub>2108</sub>                       |
| HPA-15b (Gov <sup>a</sup> ) | a/b                  | 42%                                         |                                            |                                           |
|                             | b/b                  | 23%                                         |                                            |                                           |
| HPA-16bw                    | Duv <sup>a</sup>     | <1%                                         | GP1IIa / Thr:Ile <sub>140</sub>            | C→T <sub>517</sub>                        |
| HPA-?                       | Va <sup>a</sup>      | <1%                                         | GP1IIa / ND                                | ND                                        |
| NA                          | Nak <sup>a</sup>     | 99.8% (White)<br>97% (Black)<br>96% (Asian) | CD36 (GP1V)                                | T→G <sub>1264</sub><br>C→T <sub>478</sub> |

\*Phenotypic frequencies for the antigens shown are for White populations only. Significant differences in gene frequencies may be found in Black and Asian populations.

ND = not determined; ? = antigen number not yet assigned; NA = not applicable.

after blood transfusion (PTP); and patients who have received multiple transfusions. To date, 23 platelet-specific alloantigens have been described,<sup>52</sup> including localization to platelet surface glycoprotein structures, quantification of their density on the platelet surface, and determination of DNA polymorphisms in genes encoding for them (see Table 11-1). Several others have been described serologically, but genetic polymorphisms underlying these have not yet been determined.<sup>52</sup> Many of the recognized platelet alloantigens have been implicated in cases of FNAIT and

PTP (see Chapter 23). Some also have been determined to be relevant in platelet transfusion responses (see below).

## Alloimmunization to Platelet Antigens

### Immunization to HLA Antigens

Pregnancy and transfusion account for the development of HLA alloimmunization, with exposure to leukocyte-containing blood

components resulting in high rates of antibody formation in patients receiving multiple transfusions over time. Antibodies to Class I and not Class II HLA antigens can significantly affect the recovery and survival of transfused platelets because the former and not the latter are expressed on the platelet surface. Among the Class I antigens, the HLA-A and -B markers are most important. HLA-C antigens are also present and reported to be expressed at approximately the same density as A antigens,<sup>14</sup> but with rare exceptions,<sup>53</sup> antibodies to HLA-C antigens do not appear to significantly affect transfused platelets.

The natural history of HLA sensitization in patients receiving platelet transfusions for hematologic diseases was studied in 1978,<sup>54</sup> before the advent of widespread use of leukocyte-reduced blood components. Sixty-three patients requiring transfusions of whole-blood-derived platelets were followed. By actuarial analysis, 60% of patients who were not positive for lymphocytotoxic antibodies at the beginning of the study were projected to develop them as early as 10 days after primary exposure or 4 days after secondary exposure if they had been transfused or pregnant in the past. The number of transfusions was not related to the likelihood of immunization, an observation that was confirmed in a later study.<sup>55</sup> Patients who were alloimmunized at the beginning of the study had the poorest responses; those who did not develop HLA antibodies had the best responses; and those whose antibodies developed during the period of observation had intermediate responses.<sup>54</sup> A more recent study of platelet preparation methods to reduce alloimmunization in newly diagnosed acute myelogenous leukemia (AML) patients found a similarly high HLA sensitization rate of 45% in the control group (131 patients) who received unmodified, pooled, whole-blood-derived platelet transfusions over an 8-week period.<sup>56</sup> Rates of alloimmunization in studies involving hematology-oncology patients receiving non-leukocyte-reduced blood components are summarized in Table 11-2.

The risk of HLA alloimmunization is influenced by several patient and blood component factors. Transfused patients who were exposed previously to allogeneic HLA via transfusion or pregnancy developed HLA antibodies sooner—and, in many studies, more often—than patients who were not exposed previously.<sup>56,61,63,64</sup> In the Trial to Reduce Alloimmunization to Platelets (TRAP) study,<sup>56</sup> 62% of previously pregnant women with AML receiving untreated blood components (control product) developed lymphocytotoxic antibodies compared with 33% of those who had not been pregnant or transfused previously. There is general agreement that primary alloimmunization to HLA antigens is unlikely to occur before 3 to 4 weeks after the first transfusion in patients receiving multiple transfusions, and that HLA antibodies detected sooner than this most likely represent secondary immune responses in patients with remote histories of transfusion or pregnancy.

The underlying disease for which patients require platelet transfusion also influences the rate of HLA alloimmunization. In one study, patients undergoing induction chemotherapy for AML were more likely to become alloimmunized than were

**Table 11-2.** Platelet Alloimmunization in Multitransfused Patients Receiving Non-Leukocyte-Reduced Blood Components\*

| Study                                | No. of Patients | Anti-HLA <sup>†</sup> | Anti-PSA <sup>‡</sup> | Loss/<br>Decrease in<br>Antibody |
|--------------------------------------|-----------------|-----------------------|-----------------------|----------------------------------|
| Seftel et al (2004) <sup>57</sup>    | 315             | 61/315 (19%)          |                       |                                  |
| TRAP (1997) <sup>56</sup>            | 131             | 59/131 (45%)          | 11/131 (8%)           |                                  |
| Atlas et al (1993) <sup>58</sup>     | 134             | 95/134 (71%)          |                       | 43/95 (38%)                      |
| Meenaghan et al (1993) <sup>59</sup> | 106             | 37/106 (35%)          | 45/106 (42%)          | 29/45 (64%)                      |
| Godeau et al (1992) <sup>60</sup>    | 50              | 13/50 (26%)           | 4/50 (8%)             |                                  |
| Pamphilon et al (1989) <sup>61</sup> | 49              | 20/49 (41%)           | 11/49 (22%)           | 12/20 (59%)                      |
| Murphy et al (1987) <sup>62</sup>    | 154             | 55/154 (36%)          | 5/154 (3%)            | 30/55 (54%)                      |

\*Frequency of HLA antibody and platelet-specific antibody formation in studies of patients with hematologic and oncologic diagnoses requiring repeated platelet transfusion. The final column contains frequencies of antibody loss or decline in these studies.

<sup>†</sup>HLA antibodies determined by lymphocytotoxicity testing.

<sup>‡</sup>Platelet-specific antibodies determined by a variety of methods.

patients being treated for acute lymphoblastic leukemia (ALL).<sup>65</sup> Although both groups of patients received similarly intensive chemotherapy and required roughly the same level of transfusion support, HLA antibodies developed in 44% of the AML group, compared with 18% of the patients with ALL ( $p = 0.00002$ ). The authors postulated that the difference may be attributable to either an additional immunosuppressive effect of the high-dose corticosteroids given in ALL or to a decreased immune responsiveness in patients with ALL caused by their underlying disease.<sup>65</sup> Others have corroborated this finding and have noted, moreover, that alloimmunization seems to occur sooner in patients with AML than in those with ALL.<sup>61,66</sup>

Except for prospective solid organ transplant recipients, there have been limited studies of HLA alloimmunization in patients who received transfusions of Red Blood Cell (RBC) or Whole Blood units, but interest in sensitization rates in such patients has increased, particularly with the advent of marrow transplantation for hemoglobinopathies such as sickle cell disease. Sensitization to HLA can inhibit platelet transfusion responses (see below), increasing the risk of thrombocytopenic hemorrhage in patients undergoing marrow transplantation who require multiple platelet transfusions. One study of chronically transfused sickle cell disease patients determined that 85% of those with at least 50 past red cell transfusions had evidence of platelet-reactive antibodies, predominately anti-HLA, whereas 48% of those with fewer than 50 past exposures were sensitized. HLA antibodies were not detected in those patients who had no

past exposure to transfusion.<sup>67</sup> A second study of 60 thalassemia patients examined the development of HLA antibodies and found that 32 (53%) were positive for HLA antibodies at baseline and seven more became sensitized in the follow-up period of 1 year, for a total HLA sensitization rate of 65%.<sup>68</sup> The type of blood components used for these two groups of patients may have contributed to these significant rates of alloimmunization. Details about the type of RBC transfusions provided in the first study were not provided in the report, but there did not appear to be any systematic attempts to give leukocyte-reduced units. Washed RBCs were routinely used in the second study, and these were estimated to have residual leukocyte content of  $>5 \times 10^6$  per unit. Therefore, neither study used RBC units that were leukocyte reduced to the extent that HLA sensitization would be lessened (see below).

Still other investigators have sought to document the rates of HLA alloimmunization in nonhematologic conditions requiring shorter periods of transfusion support. In a study of 117 cardiac surgery patients who were exposed to a single episode of RBC transfusion, 18% became sensitized.<sup>69</sup> In contrast, in a second study of 40 more seriously ill patients requiring placement of left ventricular assist devices as a bridge to cardiac transplant, 45% formed Class I HLA antibodies after a course of support that included a number of platelet as well as red cell transfusions. By actuarial analysis, 63% of those who received more than six transfusions of pooled whole-blood-derived platelets were projected to become HLA immunized.<sup>70</sup> This rate was strikingly similar to that of the patients with hematologic malignancies followed in the earlier study noted above.<sup>54</sup>

These studies documenting HLA alloimmunization in patients with non-hematologic-oncologic conditions suggest that apart from patients with ALL, most patients who require repeated red cell and/or platelet transfusions for treatment of their hematologic-oncologic condition are at risk of becoming sensitized to HLA at a rate similar to patients who would be assumed to have normally functioning immune systems.

Both the number and type of platelet products also have an impact on the sensitization rate. There is some disagreement in the literature regarding the importance of a dose-response relationship between platelet transfusions from different donors and the risk of alloimmunization. One study<sup>55</sup> failed to note such a relationship in a group of patients with AML receiving induction therapy. However, most of the patients in this study were exposed to more than 20 different platelet donors. Indeed, animal data<sup>71</sup> and human transfusion trials<sup>72</sup> suggest that with fewer donor exposures (ie, less than 20), there is a dose-response relationship between the number of exposures and the rate of alloimmunization. Fewer donor exposures can be achieved using apheresis platelets, which provide adequate doses of platelets from a single donor rather than pooled platelet concentrates. Alloimmunization can be delayed and perhaps reduced using this type of platelet product.<sup>72</sup>

Despite the established correlation between HLA antibodies and refractoriness to platelet transfusions (see below),<sup>73,74</sup>

lymphocytotoxic antibodies are frequently a transient complication in patients with hematologic-oncologic diagnoses requiring repeated platelet transfusions. Studies document that 17% to 67% of patients demonstrating these antibodies eventually lose them (Table 11-2).<sup>57,58,61,62,65,75,76</sup> The loss of lymphocytotoxic antibodies may be related to discontinuance of the antigenic exposure after marrow recovery or to switching of platelet transfusion support to HLA-matched homologous or frozen autologous platelets; however, it can occur despite continued exposure to whole-blood-derived platelet transfusions.<sup>62,65,75,77</sup> One interesting report<sup>58</sup> found that two-thirds of patients with decreasing anti-HLA reactivity despite continued exposure had developed antiidiotypic antibodies that reacted with the V region of anti-HLA IgG. In 36% of these patients, the serum actually inhibited binding of the patient's own prior anti-HLA to appropriate lymphocyte targets. A history of pregnancy did not affect the ability to produce these antiidiotypic antibodies. In contrast, those patients with persistently detectable anti-HLA did not develop antiidiotypic reactivity. HLA antibodies that are detected before the onset of transfusion therapy (ie, because of remote transfusion or pregnancy) tend to persist throughout the current period of transfusion therapy, whereas those antibodies that develop *de novo* during a transfusion support episode are more likely to be transient and to decrease in strength or disappear altogether despite continued exposure to allogeneic blood and platelets.<sup>62</sup>

The major route of primary HLA immunization in transfused platelets involves the donor leukocytes, and not the platelets, *per se*. In-vitro experiments showed that highly purified HLA-A2+ platelets could not induce allo-cytotoxicity in HLA-A2-negative peripheral blood mononuclear cells (PBMCs)—not even in the presence of helper HLA-A2-negative PBMCs.<sup>6</sup> Studies in humans show that when platelets devoid of white cells are transfused, primary immunization to HLA is very much delayed or does not occur at all,<sup>56,78-80</sup> whereas unmodified platelet concentrates are associated with a rate of HLA immunization ranging from 19% to 71% (Table 11-2). These observations implicate the leukocytes in both platelet and red cell transfusions as the source of primary immunization. Animal studies confirm that the production of major histocompatibility complex (MHC) antibodies requires recognition of Class II MHC molecules on donor antigen-presenting cells (APCs) by recipient T cells.<sup>81</sup> Moreover, animal studies also suggest that cell-free plasma supernatants from platelet concentrates stored without prior leukocyte reduction contain immunizing fragments thought to have been derived from white cells,<sup>82</sup> and these fragments are not significantly removed by leukocyte reduction filters.<sup>83</sup>

### Immunization to Platelet-Specific Antigens

The importance of platelet-specific antigen systems in the clinical syndromes FNAIT and PTP is undisputed and is described in Chapter 23. However, the relevance to platelet transfusion practice is controversial.

One kind of evidence implicating platelet-specific antibodies in the destruction of transfused platelets is the failure of some

transfusions of HLA-matched platelet concentrates given to patients who are refractory to whole-blood-derived platelets.<sup>78</sup> The poor responses to these platelet transfusions, despite HLA matching, indicates that other antigens, perhaps platelet-specific markers, are involved. The other kind of evidence involves the demonstration of platelet-reactive antibodies in the absence of lymphocytotoxic or anti-HLA activity. The conclusion drawn is that antibodies directed at platelet-specific antigens cause these reactions.<sup>77,78,84</sup> In contrast to antibodies to HLA antigens, the majority of platelet-specific antibodies identified using these strategies do not seem to be associated with poor transfused platelet recovery or survival.

With the advent of Phase III platelet antibody assays that detect platelet glycoprotein-specific reactions, it is now possible to assay directly the sera from multitransfused patients for platelet-specific antibodies. Using one such method, the monoclonal antibody-specific immobilization of platelet antigens (MAIPA) assay, one group documented that among 252 patients with hematologic-oncologic diagnoses receiving platelet transfusions, 20 (8%) developed platelet-specific antibodies with clear-cut specificities. The most common specificity in these patients was for HPA-5b in 10 of the 20; followed by HPA-1b in four; HPA-5a in two; and one each with HPA-2b, HPA-1a, HPA-1b plus HPA-5b, and HPA-1b plus HPA-2b.<sup>85</sup> This study confirmed earlier findings in the TRAP study; where 8% of the 530 patients formed platelet-specific antibodies, the most common being anti-HPA-1b.<sup>56</sup>

These two studies confirm the finding that many of the best-documented platelet-specific antibodies detected in patients who receive transfusions are directed against platelet antigens, the phenotypic frequencies of which are less than 30% in the blood donor population.<sup>56,69,85</sup> Therefore, it is difficult to attribute refractory responses to whole-blood-derived and/or HLA-matched platelet transfusions to these antibodies alone. Indeed, the majority of refractory patients with platelet-specific antibodies also have HLA antibodies. Alloimmunization to high-frequency platelet-specific antigens would be expected to present a major challenge in finding compatible platelets to support a patient requiring multiple platelet transfusions. Fortunately, these cases are extremely rare.<sup>69,86</sup>

Although there are a few well-documented cases of transfusion failures attributable to platelet-specific antibodies,<sup>87-89</sup> most platelet glycoprotein reactivity lacking definite specificity in transfusion recipients does not seem to influence transfusion responses.<sup>56,60,76,90</sup> One interesting report suggests that in a cohort of 106 patients who received multiple transfusions, 42% developed platelet-specific antibody, as detected by chloroquine-treated platelets in a platelet antibody screening test. Western blotting on these sera identified a number of bands to which IgG in the sera reacted, most commonly with molecular weights of 80 to 83 kD. In three of the sera the reactivity was not detected against thrombin-treated platelets, indicating that the antibodies might be directed against platelet GPV. No correlation with platelet transfusion responses was reported, and more than half of these "platelet-specific" antibodies were transient and not detected in follow-up testing.<sup>59</sup>

There have been several reports of platelet transfusion refractoriness caused by antibodies to GPIV (CD36) in patients who are GPIV deficient.<sup>34,36,40</sup> These patients are difficult to support because virtually all platelet products available for transfusion would be incompatible (GPIV positive). In several reports, GPIV-negative platelets were obtained by large-scale screening of donor populations with a higher frequency of GPIV deficiency (African), and transfusion of those platelets resulted in good platelet increments for the patients.<sup>40-42</sup>

### Immunization to Blood Group Antigens

Several studies have documented the effect of ABO incompatibility on platelet transfusion therapy. In an early report of 91 alloimmunized thrombocytopenic patients receiving 389 transfusions, 24-hour transfused platelet recovery was reduced by 23% in ABO-incompatible donor-recipient pairs (donor-group A, B, or AB; recipient-group O, B, or A).<sup>91</sup> In the aggregate, patients receiving multiple platelet transfusions have been thought to demonstrate a statistically significant but clinically unimportant decrease in platelet recovery after transfusion of ABO-incompatible platelets (especially group A). In a subset, however, as many as 20% of group O patients could develop severe refractoriness to group A platelets.<sup>20,22,92</sup> Failure to respond to HLA-matched platelet transfusions in the absence of nonimmune clinical factors should prompt the clinician to examine the ABO group of the recipient and of the donors to determine whether ABO incompatibility might be responsible for the unexpected poor responses.

Heal et al<sup>93</sup> have examined the importance of ABO blood groups in platelet transfusion therapy. Forty patients with hematologic diseases receiving platelet transfusions were randomly assigned to receive either ABO-identical or ABO-unmatched platelets. The responses in the group of patients receiving ABO-unmatched transfusions (that is, when either the recipient would be expected to have isoagglutinins to the ABH antigens on the transfused platelets, or the donor had such antibodies directed at the recipient's blood type) were significantly worse than those observed in patients receiving ABO-identical platelet transfusions. Analysis of the first 25 transfusions in each group showed a significantly better response in the ABO-identical arm [mean corrected count increment (CCI), 6600 vs 5200;  $p < 0.01$ ]. This effect was most important in the first 10 transfusion episodes and tended to predict subsequent alloimmunization and refractoriness to platelet transfusions.<sup>93</sup> This finding seemed to be in conflict with earlier reports in which a minor, clinically insignificant impact of ABO mismatching was observed. The newer data were then reanalyzed using the earlier definitions of ABO "compatibility" (the patient lacks isoagglutinins to recipient ABO antigens) and "incompatibility" (the patient has isoagglutinins reactive with donor ABO antigens). In the reanalysis, no benefits of ABO compatibility were detected,<sup>94</sup> suggesting that there is a significant negative impact of both major and minor ABO incompatibility in platelet transfusions. An increased frequency of refractoriness in patients receiving ABO-unmatched platelet

transfusions was also observed in a study of 26 patients (69% vs 58%;  $p = 0.001$ ).<sup>95</sup>

The mechanism for platelet destruction in platelet ABO-incompatible transfusions (the recipient has isoagglutinins against donor ABH) is not difficult to ascertain. Presumably, IgM and IgG anti-A or anti-B in the recipient interact with A and B substances on the transfused platelets, resulting in their premature exit from the circulation. Explanations offered for the biphasic survival curves of ABO-incompatible platelets include: 1) the elution of a portion of group A substance from the platelet surface; 2) the nonhomogeneous distribution of group A substance on donor platelets with resultant rapid destruction of the subpopulation with highest expression; and 3) secondary injury to a subset of transfused platelets caused by the reaction between anti-A isoagglutinins and A red cells in the platelet concentrate.<sup>18,19</sup>

The suboptimal response of the *plasma*-incompatible transfusions (the donor has isoagglutinins against recipient ABH) is more difficult to explain. One report postulates that immune complexes involving soluble recipient ABH substance and donor A or B antibodies form. These immune complexes secondarily interact with the transfused platelets via the  $\text{Fc}\gamma\text{RII}\alpha$  receptor, or the complement receptors  $\text{cC1q-R}$  and  $\text{gC1q-R}$ , and mediate their destruction.<sup>96</sup> Some experimental evidence supports this theory, in that anti-A has been detected in an immune complex fraction of group A recipient plasma after transfusion with group O platelets, and these immune complexes bind to IgG  $\text{Fc}\gamma\text{RII}\alpha$  and the  $\text{cC1q-R}$  and  $\text{gC1q-R}$  receptors on group O platelets.<sup>96</sup> Indeed, in at least one study, plasma-incompatible platelet transfusions were even less effective than platelet-incompatible transfusions.<sup>93</sup> Immune complexes involving other plasma proteins such as C2, C4, albumin, and fibrinogen likewise have been implicated in refractory responses to platelet transfusion.<sup>96-98</sup>

Adverse effects of ABO-incompatible platelet transfusions may extend beyond reducing platelet transfusion increments and stimulating alloimmunization. In one retrospective cohort study of cardiac surgery patients, those patients who received mismatched ABO platelets were compared with those who received ABO-identical platelets. The mismatched group experienced significantly longer hospital stays, more days of fever, increased health-care costs, and more red cell transfusions. Other negative outcomes including mortality and time in the intensive care unit were also increased in the mismatched group, but these differences failed to reach statistical significance. The authors postulated that the negative effects of ABO-mismatched platelet transfusions were again related to immune complexes that stimulated inflammatory pathways, leading to increased postoperative morbidity in these patients.<sup>99</sup> Although intriguing, these findings have yet to be confirmed by other groups. In fact, a later retrospective study involving more cardiac surgery patients failed to identify negative effects of ABO-mismatched platelet transfusions.<sup>47,100</sup>

An indisputable risk of ABO plasma-incompatible transfusions, particularly those involving group O donors and non-group-O

recipients, is acute hemolytic transfusion reaction caused by high-titer isoagglutinins in the donor plasma. With the marked shift from pooled whole-blood-derived platelet concentrates to apheresis platelets, the risk of transfusing large volumes of ABO-incompatible plasma with potent ABO isoagglutinins is increased. Many blood providers have taken steps to alleviate this risk by reducing the volume of incompatible plasma or screening group O donors for high-titer isoagglutinins.<sup>101</sup>

## Transfusion Refractoriness

*Alloimmunization* is an immune response in a recipient stimulated by foreign donor cells. In the platelet transfusion setting, these responses involve the production of antibodies directed at donor platelets. *Platelet refractoriness* describes a clinical condition in which patients do not achieve the anticipated platelet count increment from a platelet transfusion. It is possible to be alloimmunized to platelet antigens without being refractory to platelet transfusions, and also to be refractory to platelet transfusions without being alloimmunized. *Alloimmune refractoriness* occurs when the level of alloimmunization, as measured by the breadth of antibody response to platelet antigens, is sufficient to affect the majority of randomly selected platelet products.

The detection of alloimmunization is straightforward using standard laboratory techniques to detect antibodies in the patient's serum reactive with HLA or platelet-specific antigens. In contrast, the definition of the refractory state is less precise.<sup>102,103</sup> A standard dose of platelets (six units of pooled whole-blood-derived platelet concentrates or one apheresis platelet unit) generally increases the platelet count by about 5000 to 7000 platelets/ $\mu\text{L}$ /unit in a 70-kg adult. This would result in a posttransfusion increment of about 30,000 to 40,000 platelets/ $\mu\text{L}$  1 hour after platelet transfusion.<sup>102</sup> The TRAP study defines the refractory state as a CCI [normalizing transfusion responses for patient blood volume estimated using body surface area (BSA) and platelet dose] of <5000 after two sequential ABO-compatible platelet transfusions.<sup>56</sup> Another measure of platelet transfusion response is the percent platelet recovery (PPR). Similar to the CCI, the PPR uses platelet dose and patient blood volume; the latter is estimated using the patient's body weight in kg rather than BSA. Studies in normal autologous platelet donors show an average 1 hour PPR of approximately 66%.<sup>74</sup> Recovery less than 20% to 30% at 1 hour after the transfusion indicates a refractory response.<sup>103</sup>

The PPR and CCI have been criticized as measures of posttransfusion platelet response because both calculations, which correct for patient size and dose of platelets, fail to provide information about the impact of these two variables on the posttransfusion platelet count increment.<sup>104</sup> For example, a small dose of platelets given to a large patient might result in an acceptable PPR or CCI but a poor absolute platelet count increment. In order to better examine the impact of patient size and platelet dose, as well as other factors that affect the quality of the platelets



(storage time, postcollection manipulations, etc) regression analysis of posttransfusion platelet increments is suggested. It is also recommended by the same authors that because both the CCI and PPR calculations are biased in favor of platelet preparation techniques that provide fewer platelets, neither should be used to define platelet refractoriness. Regardless of which method is used, the actual platelet increment in patients who are highly refractory because of alloimmunization is extremely small to negligible.

It is important to recognize that refractoriness does not necessarily imply alloimmunization. Indeed, the major cause of refractoriness to platelet transfusion is not alloimmunization, but rather nonimmune factors that result in shortened platelet survival and/or markedly decreased platelet recoveries in patients who receive transfusions.<sup>74,105</sup> Multiple linear regression analysis has been used to demonstrate a number of factors related to clinical refractoriness including HLA alloimmunization as well as splenomegaly, amphotericin therapy, disseminated intravascular coagulation, or recent allogeneic marrow transplantation.<sup>106</sup> Other studies have also demonstrated factors with negative effects on transfused platelets such as sepsis, fever, and drugs.<sup>107,108</sup> Individual patients may have significantly different responses to the same nonimmune causes of refractoriness, with some patients experiencing minimal impact and others having markedly impaired response to platelet transfusions. Some patients, particularly those with multiple clinical complications (sepsis, fever), appear to respond poorly to platelets that are approaching the end of the recommended storage interval (5 days). Such patients may experience an improvement in their platelet response after receiving “fresh platelets”—platelets that were collected less than 48 hours earlier.<sup>102,109</sup>

A cause of non-alloimmune refractoriness that can be overlooked in the infected, neutropenic, hematology-oncology patient is development of drug-dependent platelet-reactive antibodies. Vancomycin, a drug often used to treat serious gram-positive bacterial infections in such patients, has been implicated. Drug-dependent platelet-reactive antibodies should be suspected in refractory patients when there is no evidence of alloimmunization or they fail to respond to HLA-selected platelets, and the refractory responses are temporally related to therapy with a drug.<sup>110,111</sup>

## Treatment of the Refractory Alloimmunized Patient

The refractory state in which patients fail to benefit from whole-blood-derived platelet transfusions develops in 13% to 100% of patients receiving multiple transfusions.<sup>56,61,112,113</sup> The refractoriness, if related to HLA immunization, can be transient or persistent. Several approaches can be considered to provide such patients with adequate platelet support—including provision of HLA-matched platelets, platelets selected by crossmatch tests, and maneuvers to reduce alloimmunization.

## Platelet Selection

### HLA-Selected Platelet Transfusions

A standard approach to supporting a patient who is refractory to whole-blood-derived platelet transfusions is to supply HLA-matched apheresis platelet concentrates. Because the primary cause of immune refractoriness to platelet transfusion is alloimmunization to Class I HLA antigens, it follows that avoidance of incompatible HLA specificities should result in a more successful platelet transfusion response. In practice, up to 90% of alloimmune refractory patients benefit from an HLA-matched product. This was first demonstrated in a study that showed patients who were refractory to platelets from unselected donors could be successfully supported by HLA-matched family member platelet transfusions.<sup>114</sup>

Certain “private” HLA antigens can be segregated into so called cross-reactive groups (CREGs), defined by antibodies directed against shared “public” determinants.<sup>115</sup> Indeed, these shared determinants are the basis for the cross-reactivity and are different than the private determinants, which account for the highly polymorphic HLA system. Selection of platelet donors with antigens in the same CREGs as the antigens in the patient, so called cross-reactive antigens, was demonstrated to be nearly as successful in supporting alloimmune platelet refractory patients as HLA-identical transfusions.<sup>116</sup> This appeared to be due to the relative inability of the patient’s immune system to recognize these cross-reactive antigens as different, thereby greatly increasing the number of potentially successful platelet donors in a given pool.<sup>117,118</sup>

A disadvantage of relying on HLA-matched platelets, even using selective mismatching with CREG associations, is that a pool of 1000 to 3000 or more HLA-typed potential apheresis donors is generally necessary to find sufficient HLA-compatible matches to support a typical patient.<sup>119</sup> Moreover, donor selection on the basis of HLA type can lead to the exclusion of donors whose HLA types, although different from that of the recipient, may still be effective.<sup>120</sup>

In alloimmune refractory patients, the best increases in CCI occur with the subset of grade A and B1U or B2U HLA-matched platelets, but platelets mismatched for some antigens (eg, B44, 45) that are poorly expressed on platelets can also be successful.<sup>120</sup> Although alloimmunized patients with high panel-reactive antibody (PRA) values benefit only from platelet products that lack any incompatible Class I antigens (match grades A, B1U, B1X, B2U, B2UX, see Table 11-3), patients with lesser degrees of sensitization can sometimes benefit from less well-matched platelets. In one study, 73% of HLA single-antigen mismatched platelet transfusions (grade C match) were successful when provided to patients with PRA values less than 60%.<sup>121</sup> On the basis of these data, some experts suggest extending donor searches for alloimmunized patients to include single-antigen mismatches (grade C matches), particularly if the PRA is less than 60%.

Even with the additional donors that the above cited studies may make available, HLA-matched platelets are frequently unavailable for many refractory patients. Use of the patient’s HLA

**Table 11-3.** Classification of Donor/Recipient Pairs on the Basis of HLA Match

|      |                                                                                                     |
|------|-----------------------------------------------------------------------------------------------------|
| A    | All four antigens in donor identical to those in recipient.                                         |
| B1U  | Only three antigens detected in donor; all present and identical in recipient.                      |
| B1X  | Three donor antigens identical to recipient; fourth antigen cross-reactive with recipient.          |
| B2U  | Only two antigens detected in donor; both present and identical in recipient.                       |
| B2UX | Only three antigens detected in donor; two identical with recipient, third cross-reactive.          |
| B2X  | Two donor antigens identical to recipient; third and fourth antigens cross-reactive with recipient. |
| C    | One antigen of donor not present in recipient and non-cross-reactive with recipient.                |
| D    | Two antigens of donor not present in recipient and non-cross-reactive with recipient.               |

antibody specificity as an additional basis for selection of platelet products has been demonstrated to increase the numbers of potentially compatible donors. Antibody specificity prediction (ASP), allows procurement of platelet products from donors who lack HLA antigens to which the patient has raised an antibody. Such platelet donors often have frank mismatches for some or all of the Class I antigens in the refractory patient. One study compared ASP platelets to those selected by standard HLA matching criteria and by platelet crossmatching. HLA-matched, crossmatched, and ASP-selected platelet transfusions were found to have similar platelet recoveries, while randomly selected control platelets had significantly lower PPR. For 29 alloimmunized patients, the mean number of potential donors found in a file of 7247 HLA-typed donors was only six when grade A HLA matches were required and 39 when BU matches were added. However, 1426 potential donors (20% of total) were identified by the ASP method. The authors suggest that careful HLA antibody specificity identification could greatly enhance the number of potential donors by identifying nonmatched products that lack these HLA antigens.<sup>122</sup> Other investigators using a computerized analysis of the lymphocytotoxicity (LCT) assay for private and public HLA Class I epitopes in platelet recipients confirmed that there is value in carefully identifying HLA antibody specificities, allowing selection of many more donors by simply avoiding the HLA antigens against which the antibodies are directed.<sup>123</sup>

Regardless of the method planned to select HLA-compatible platelets for alloimmune refractory patients, the HLA type of the patient should be determined before myeloablative therapy, and HLA antibody screening should be obtained periodically (preferably weekly) so that HLA-selected platelets can be provided when and if alloimmune refractoriness is diagnosed.

In a small number of patients who fail to respond to HLA-matched platelets and for whom no other nonimmune explanation can be found for refractoriness, platelet-specific

alloantibodies may be the cause of the poor platelet increments. Approximately 7% to 8% of platelet multitransfused patients develop platelet-specific antibodies.<sup>56,124</sup> Many of these patients are also alloimmunized against HLA. One recent report described six patients who were highly alloimmunized to HLA but also had human platelet alloantibodies to HPA-1b or HPA-5b.<sup>125</sup> These patients were successfully supported with a pool of HLA-matched platelets that were typed for the HPA antigens.

### Platelet Crossmatching

Although the use of HLA-matched and selectively mismatched donors provides support for the majority of patients who are alloimmune refractory, there are limitations to this strategy. As many as 20% to 25% of refractory patients fail to respond adequately to HLA-matched platelets. In the absence of nonimmunologic clinical factors that can decrease platelet transfusion recovery and survival, these failures might be explained by ABO incompatibility, platelet-specific antibodies, and undetected HLA incompatibility.<sup>126</sup> These immune causes of transfusion failure cannot be addressed with HLA matching alone. In addition, many facilities do not have access to adequately sized HLA-typed donor files, so that alternative methods of selecting compatible platelets for alloimmune refractory patients must be considered.

Many platelet and leukocyte antibody detection methods have been assessed as platelet compatibility tests. One method that has gained wide acceptance is the commercially available solid-phase red cell adherence (SPRCA) assay. The test is rapid and sensitive, particularly for the detection of HLA antibodies, and is therefore suitable for routine platelet crossmatching.<sup>127,128</sup> Good correlation between test results and posttransfusion platelet counts have been achieved with the SPRCA assay.<sup>128-131</sup>

To date, few studies have compared the efficacy of HLA matching to that of platelet crossmatching for supporting alloimmune refractory patients. One multicenter study<sup>132</sup> compared HLA selection and platelet crossmatching using three different techniques—the indirect immunofluorescence test for platelets, an enzyme-linked immunosorbent assay (ELISA), and a radioimmunoassay were used to test apheresis platelet concentrates. At least one pair of apheresis platelet components was transfused to each patient. One unit was selected by HLA matching and the other by crossmatching. The results of the two donor selection methods were compared. Although the difference was not statistically significant, HLA-selected transfusions were slightly more successful than the crossmatch-selected transfusions when 1-hour posttransfusion recoveries were compared. However, after 24 hours the HLA-selected transfusions were significantly more successful. This was even more apparent when only A and BU matches were considered. The authors concluded that when HLA-typed donors are available, A or BU matches were preferable to randomly chosen products selected by any of the crossmatch methods used in their study. The quality of the HLA match is paramount to the transfusion success rate as shown in a study in which HLA matching apparently did not improve on the success rate of crossmatch-selected platelets.<sup>130</sup> Here, however,

the HLA-selected platelets were seldom of A or BU match grade with the recipients.

The degree to which a patient is alloimmunized has an impact on the success of platelet transfusions selected by crossmatching assays. Using platelet concentrates from whole blood units and a radiolabeled antiglobulin technique, one study found that 70% of transfusions selected by the test were successful in supporting moderately alloimmunized patients.<sup>133</sup> In contrast, others found that only 41% of transfusions selected by either an ELISA or the SPRCA test were successful in a highly alloimmunized group of leukemia patients.<sup>129</sup> Moreover, only 15% to 24% of platelet concentrate segments tested were negative in these tests, indicating that many whole-blood-derived platelet concentrates had to be screened in order to identify sufficient compatible platelets to pool for the transfusions. In the latter study, the most compatible platelet products selected by crossmatching were less successful in supporting the refractory patients than were HLA-matched platelets (mean CCI of 4300 to 9500 with “crossmatched-compatible” platelets; mean CCI of 17,100 with HLA-matched platelets).<sup>129</sup>

A more recent report of an automated version of the SPRCA assay demonstrated how large numbers of platelet concentrates could be routinely tested to identify compatible pools of whole-blood-derived platelets for refractory patients. In this study, a daily inventory of 100 to 120 whole-blood-derived buffy coats were assessed to provide platelet support for 40 consecutive alloimmune refractory patients.<sup>134</sup> Platelets collected from routinely obtained donor samples, rather than from the concentrates themselves, were tested with patient serum. Using this system, up to 94 different platelet samples could be tested for each patient. Buffy coat platelets from five or six donors whose platelets yielded negative results were then pooled and transfused to the patient. The authors were able to demonstrate significant improvement in both 1-hour and 18- to 24-hour increments with the crossmatch-negative pools compared with random pools given in the month before the patients’ meeting the criteria for refractoriness in the study (two consecutive 1-hour post-transfusion CCIs <5000).

The cost of the kits for achieving each “successful” platelet increment (posttransfusion platelet count >10,000/ $\mu$ L) was about 450 euros. This did not include any costs for labor or administration of the crossmatching program. The authors noted that although the automated crossmatching strategy allowed rapid identification of compatible platelet pools from available inventories, a larger study, preferably a randomized controlled trial of this method vs HLA matching, would be necessary to confirm the value of this approach and more accurately capture the costs involved.

Experience using both the LCT assay and the SPRCA for analyzing antibodies to HLA and platelet antigens has been reported to aid in identifying patients who will benefit from either HLA-selected or crossmatch-selected platelets.<sup>135</sup> With the LCT assay, investigators found that if the PRA was <70%, adequate transfusion responses were seen in approximately 80% of

transfusions that were selected simply by HLA criteria (ASP and CREGs). However, when the PRA was >70%, only about 25% of patients did well. When analyzing the results using crossmatching, patients with PRAs <80% did well with crossmatch-selected platelets; but when the PRA was 80% to 100%, there were many failures with the crossmatch-compatible products. It was suggested that at high levels of alloimmunization, crossmatching misses significant antibodies. A recent report compares the monoclonal antibody-specific immobilization of platelet antigens (MAIPA) assay to the LCT test for the detection of HLA antibodies. This study showed the MAIPA assay to be more sensitive, detecting apparent HLA antibodies missed by the LCT assay. Moreover, these MAIPA-positive, LCT-negative HLA antibodies may be clinically relevant and affect the posttransfusion platelet count increment.<sup>136</sup> The advent of more sensitive methods for screening patients for HLA antibodies, when used to identify patients who are alloimmune refractory to platelet transfusions, may prove to be superior to the LCT assay for this purpose.<sup>137</sup>

With the considerable effort and expense entailed in their procurement, HLA- or crossmatch-selected platelet products should be reserved for those refractory patients who have definite evidence of alloimmunization either by HLA or platelet antibody testing. In the latter case, alloimmunity can be established in the context of performing a platelet crossmatch test if at least some of the platelet products tested with patient serum are reactive in the assay. In practice, when refractoriness is first recognized during use of random donor platelets, provision of a few HLA-selected products may be warranted before the results of definitive testing to document alloimmunization are available. In the event that such testing fails to document alloimmunity (ie, no HLA- or platelet-reactive antibodies are identified), then support with randomly selected platelet products should resume.

The one report of improving platelet transfusion increments by use of crossmatch-compatible platelets without establishing that the refractory patients were indeed alloimmunized, actually excluded all patients who did not have evidence of platelet-reactive antibody in the crossmatch test and therefore was consistent with the recommendation to document alloimmunity to platelets before committing patients to receive either HLA-matched or crossmatched platelets.<sup>138</sup>

Although the incidence of platelet-specific antibodies causing patients to be refractory to most or all attempted platelet transfusions is very small, this possibility should be investigated when most of the attempted crossmatches are positive or when HLA-matched transfusions fail. If platelet-specific antibodies are present, donors of known platelet antigen phenotype or family members, who may be more likely to share the patient’s phenotype, should be tested.

### Overcoming Established Alloimmunization

Strategies attempting to reverse established alloimmunity to HLA antigens have met with only limited success to date. Previously attempted methods include: 1) methods to temporarily block the immune-mediated destruction of platelets; 2) suppression

of the immune response to decrease the production of relevant antibodies; and 3) provision of modified platelets or alternative (nonplatelet) hemostatic compounds.

### Blocking Immune Destruction of Platelets

Several studies investigated the utility of high-dose intravenous immunoglobulin (IVIG) to increase the platelet count in association with transfusion in refractory patients. The results of these studies have been highly variable.<sup>139-141</sup> Initial uncontrolled trials were conducted with very small numbers of patients using whole-blood-derived platelet transfusions. Studies using somewhat larger numbers of patients have been inconclusive. One study treated 11 refractory patients with 0.4 g/kg/day for 5 days with no benefit in the 1-hour posttransfusion recoveries.<sup>139</sup> Another found a modest beneficial effect of IVIG in a randomized placebo-controlled trial with 12 alloimmunized thrombocytopenic patients.<sup>140</sup> The same donor platelets were used before and after treatment with IVIG. The posttreatment 1-hour CCIs in the IVIG group (seven patients) were significantly greater than in the five patients receiving placebo treatment—8413 and 1050, respectively. However, by 24 hours after the transfusion, there was no residual benefit of IVIG. The authors concluded that the use of IVIG could not replace HLA-matched platelet transfusions in supporting alloimmunized refractory patients.

Using very high-dose (6 g/kg) IVIG, other investigators could reverse the refractory response in some patients who failed to respond to HLA-selected LCT-compatible platelet transfusions.<sup>141</sup> Before treatment with IVIG, the patients had been refractory to HLA-matched platelet products. Following therapy, 13 of 19 patients responded with improvement in 1-hour posttransfusion platelet count increment using HLA-matched platelets. Those patients with PRAs <85% responded better than those who were more highly alloimmunized.

After initial reports of success with IVIG to improve platelet responses in immune refractory patients, other investigators examined the role of Rh Immune Globulin (RhIG) infusions in preventing refractory responses from developing. In this randomized control trial, patients treated with weekly intravenous RhIG infusions had rates of refractoriness similar to those treated with placebo, indicating that this form of reticuloendothelial system blockade was not helpful in preventing refractory responses.<sup>142</sup>

### Suppression of the Immune Response to Decrease Antibody Production

Other strategies evaluated to overcome the platelet refractory state include use of vinblastine-loaded platelet transfusions,<sup>143</sup> treatment with cyclosporin A,<sup>144</sup> immunoabsorption using staphylococcal protein A columns, and plasmapheresis.<sup>145,146</sup> Each strategy has had some limited success.

### Provision of Modified Platelets or Alternative (Nonplatelet) Hemostatic Compounds

A novel approach has been developed involving transfusion with acid-treated whole-blood-derived platelets. Treatment of platelets

with citric acid modifies HLA Class I antigens on the platelet surface, making them less recognizable to the immune system.<sup>4,5</sup> A small number of patients have been treated with such products but with very limited success and studies are still ongoing.

Several potential platelet substitutes are currently being investigated. These include lyophilized platelets, infusible platelet membranes, thromboerythrocytes, and thrombospheres. Whether these will have clinical relevancy remains for future investigation.<sup>147,148</sup>

The role of platelet growth factors in the treatment of patients who have undergone highly myeloablative therapy is not totally defined. Studies support the use of both interleukin (IL)-11 and recombinant human thrombopoietin in shortening the duration and blunting the severity of thrombocytopenia after chemotherapy. However, toxicity and the development of thrombopoietin neutralizing antibodies have halted trials using the latter agent.<sup>149</sup> Less success has been demonstrated with these agents in the setting of myeloablation with stem cell transplantation. These drugs may play more of a role in preventing rather than managing platelet refractoriness because responsive patients would require fewer platelet transfusions. Currently, only one product, recombinant human IL-11 (Neumega, Genetics Institute, Cambridge, MA), is licensed in the United States for the prevention of severe thrombocytopenia and the reduction of the need for platelet transfusion following myelosuppressive but not myeloablative chemotherapy in patients with nonmyeloid malignancies.<sup>150,151</sup>

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## Prevention of Alloimmunity

Alloimmunization to HLA antigens appears to be transient in some patients; however, for most patients, once the alloimmune refractory state is established, it is very difficult to reverse. Therapy for the refractory state is expensive and frequently requires more transfusions than therapy for patients who are not refractory.<sup>152,153</sup> Therefore, attention has turned to prevention of alloimmunization as a more practical way to ensure continued successful platelet support. Figure 11-2 represents an approach to managing platelet transfusions that encompasses both prevention of and platelet selection for refractoriness to platelet transfusions.

Several strategies have been used to prevent alloimmunization to platelet products. Antigenic exposure can be limited by the use of apheresis platelet transfusions. Both animal studies and trials involving humans have shown that HLA alloimmunization can at least be delayed (if not reduced) by the provision of apheresis platelets. Apheresis platelet products provide adequate platelet doses for adults from single donors rather than pools of whole-blood-derived platelet concentrates, which typically expose patients to five to eight different donors.<sup>72</sup> Indeed, the majority of platelet transfusions in the United States are now provided from apheresis collections, in part in order to reduce the rates of alloimmunization and refractoriness.<sup>154</sup>

A second promising method is treatment of blood components with ultraviolet B (UVB) irradiation.<sup>155</sup> UVB irradiation prevents the interaction of donor dendritic cells with recipient T

### Algorithm for Managing Platelet Support

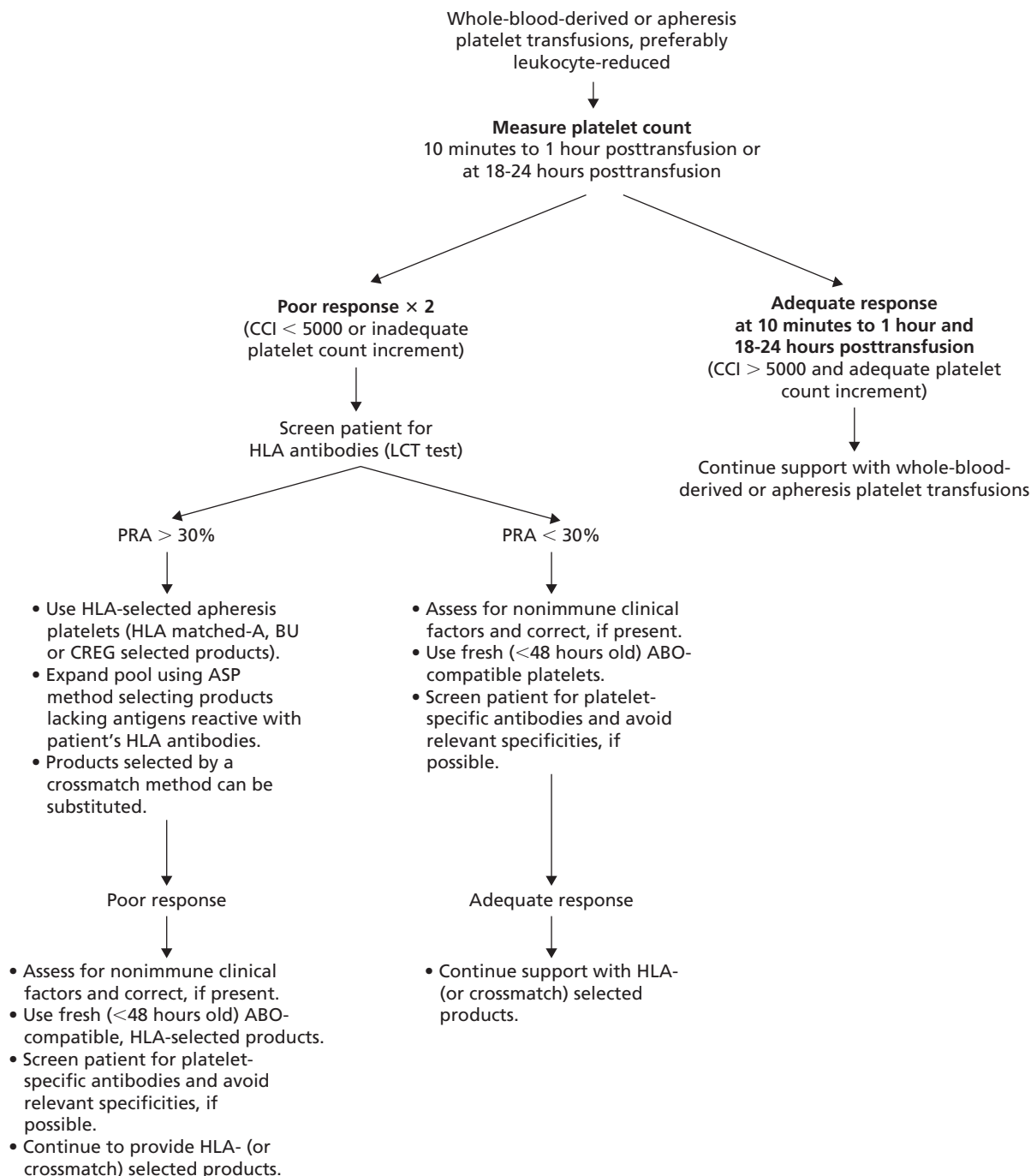


**Figure 11-2.** Algorithm for managing platelet support.

lymphocytes, which is the major route of primary HLA alloimmunization to platelet transfusions. Additional studies have provided evidence that the inability of donor and recipient immune cells to interact following UVB irradiation may indicate a state of immunologic tolerance to a blood transfusion. Preliminary studies with UVB-treated platelets in thrombocytopenic patients

with cancer demonstrated a reduced rate of HLA antibody formation. The TRAP study found that UVB irradiation could reduce the incidence of alloimmunization and platelet refractoriness.<sup>56</sup> Despite its effectiveness in reducing alloimmunization, UVB irradiation of platelet products is currently not approved for use in the United States.

Because the major pathway of primary alloimmunization against HLA Class I involves antigen presentation in the context of HLA Class II molecules on donor APCs,<sup>81</sup> significant work has centered on removing leukocytes from the donor blood components as a means of preventing alloimmunization. Clinical trials have demonstrated that reduction of leukocytes to a level  $<5 \times 10^6$  in blood components is sufficient to reduce the risk HLA alloimmunization.<sup>56,78-80</sup> Removal of leukocytes is most often accomplished by passing the components through third-generation leukocyte reduction filters. These filters remove the leukocytes by a combination of adhesion and pore size and are capable of producing a three- to four-log reduction in leukocytes. This process results in products that easily meet the Food and Drug Administration's requirement for leukocyte-reduced blood components ( $<5 \times 10^6$  total leukocytes). Although such filters can be used at the bedside, the preferred method is prestorage leukocyte reduction in the laboratory. The latter allows leukocyte-reduced blood components to be manufactured according to good manufacturing practice regulations with well-defined quality control. It also prevents leukocyte fragments, shown to be capable of passing through filters and sensitizing recipients in animal studies, from accumulating in stored blood components.<sup>82,83</sup> Leukocyte reduction of platelet products can be accomplished using either of two techniques. With apheresis technology (by which the equivalent of six whole-blood-derived platelet concentrates can be obtained from a single donor) the product is leukocyte reduced as it is collected. Alternatively, pools of whole-blood-derived platelet concentrates can be filtered before a 5- to 7-day storage period.

The majority of trials to date support the use of leukocyte reduction to reduce alloimmunization to blood components. In 1998, 18 clinical trials (some randomized controlled and some nonrandomized) using leukocyte-reduced red cells and platelets to prevent alloimmunization were reviewed.<sup>156</sup> Fourteen of the trials showed positive results using leukocyte-reduced blood to prevent HLA alloimmunization and platelet refractoriness, whereas three of the trials showed no benefit. In addition, a recent meta-analysis of eight randomized controlled trials demonstrated a clear, protective effect of white cell reduction in the prevention of HLA alloimmunization and platelet refractoriness.<sup>157</sup>

The most definitive clinical trial demonstrating the importance of leukocyte reduction in the prevention of alloimmunization and refractoriness to platelet transfusion was the TRAP study.<sup>56</sup> This trial compared four groups of patients: 1) patients receiving pooled platelet concentrates (control); 2) patients receiving leukocyte-reduced (filtered), pooled platelet concentrates; 3) patients receiving UVB-irradiated pooled platelet concentrates; and 4) patients receiving filtered platelets obtained by apheresis. All patients received leukocyte-reduced red cell components. Evidence of alloimmunization (by LCT assay) developed in 45% of the control group compared with 17% to 21% in the treated groups ( $p \leq 0.0001$  for each treated group as compared with controls). There were no differences among

the treated groups in rates of alloimmunization. In the control group, 13% of patients became refractory compared to 3% to 5% in the treated groups ( $p \leq 0.03$  for each treated group as compared with controls). Individual treatments did not differ.

Several other important findings were noted in the TRAP study: 1) Clinical outcomes were similar in all four groups of the study with no significant difference in the incidence of deaths caused by hemorrhage. 2) The incidence of refractoriness to platelet transfusions was low in the control group relative to previous reported studies. 3) Patients who had never been pregnant had a lower risk of developing refractoriness than those who had previous pregnancies. 4) Only about 8% of the patients in the study developed platelet-specific antibodies. The treatment groups had no impact on this type of immunization. 5) The presence of platelet-specific antibodies did not correlate with refractoriness. 6) A small percentage of patients in all groups were refractory to platelet transfusions yet did not have lymphocytotoxic antibodies. Most likely, these patients had either clinical factors or drug-related causes of refractoriness.

A more recent, large, single-institution, retrospective study was reported from Canada, where universal leukocyte reduction (ULR) of blood components was instituted between 1997 and 1999.<sup>57</sup> The study examined rates of alloimmunization and refractoriness in cohorts of patients undergoing chemotherapy for acute leukemia or stem cell transplantation before and after the introduction of ULR. Investigators found that alloimmunization to HLA, alloimmune refractoriness, and the need for HLA-matched platelets were all significantly reduced in the ULR cohort. Even taking into account the lower numbers of platelet transfusions given to the ULR patients, which likely resulted, in part, from changing the platelet transfusion trigger from 20,000/ $\mu$ L to 10,000/ $\mu$ L, leukocyte-reduced transfusions remained a significant independent variable driving the lower rates of alloimmunization and refractoriness in this patient population.

From the studies cited above, it is clear that both leukocyte reduction and UVB irradiation are capable of reducing the incidence of alloimmunization and platelet refractoriness. At the present time, leukocyte reduction of blood components is the most practical means of reducing the incidence of alloimmune platelet refractoriness and should be recommended for all patients at risk.

The identification and characterization of platelet antigens together with increased understanding of immunologic responses to these markers have been crucial in developing optimal platelet transfusion practices. Our understanding of the causes and mechanisms of both the immunologic and nonimmunologic aspects of the refractory response allows for improved strategies in the care of the potentially refractory patient.

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## Summary

Platelets express a variety of immunogenic markers on the cell surface. Some of these antigens are shared with other cell types

as in the case of HLA antigens, which are shared with virtually all nucleated cells in the body. Other antigens are observed to be essentially platelet specific. This chapter has reviewed the antigens expressed on the platelet, the various patterns of alloimmunization to these antigens, and the impact on platelet transfusion responses. Strategies to treat and prevent alloimmunization were also summarized.

## Disclaimer

The authors have disclosed no conflicts of interest.

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# 12

## Preparation, Preservation, and Storage of Platelet Concentrates

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Platelets may be obtained for transfusion by centrifugation of 450 to 500 mL of whole blood drawn in citrate-based anticoagulants or by various apheresis instruments that selectively collect platelets over 50 to 100 minutes. In the United States, platelets collected by either method are suspended in autologous plasma at an average concentration of  $1500 \times 10^9/\text{L}$ , six times the mean blood platelet concentration. Modern manufacturing techniques isolate 70% to 75% of the platelets from a whole blood donation ( $0.6\text{--}1.2 \times 10^{11}$ ).<sup>1</sup> Pooling of 4 to 6 whole-blood-derived (WBD) units provides a single transfusable adult dose. Alternatively, high-efficiency apheresis separators are able to harvest 65% to 75% of platelets from the 2 to 6 L of blood cycled through the machine ( $3\text{--}14 \times 10^{11}$ ), resulting in as many as three adult doses.<sup>2</sup>

Apheresis units represented approximately 86% of the platelet concentrates (PCs) transfused in the United States in 2006.<sup>3</sup> This is because of their convenience (pretransfusion pooling is not required) and because of improved safety from superior screening technology for bacterial contamination. Whole-blood-derived units, in some areas, have been relegated to a supplementary role when apheresis capacity is suboptimal or when cost-cutting measures are necessary (WBD platelets are more economical to produce).<sup>4</sup> However, because WBD pool-and-store platelet technology has now been licensed by the Food and Drug Administration (FDA), prestorage-pooled WBD platelets are also being tested for bacterial contamination employing the same culture methods used for apheresis platelets.

### Preparation of Platelets from Whole Blood—the Platelet-Rich Plasma Method

Until the late 1960s, whole blood donations had been placed on ice or refrigerated at 1–6°C since the cold sensitivity of platelets

was not appreciated. Following the introduction of plastic containers in the late 1940s, platelets were transfused primarily as platelet-rich plasma (PRP), directly infused after soft centrifugation to separate the infranatant red cells.<sup>5</sup> Further attempts to concentrate platelets from PRP resulted in an irreversibly clumped mass at the bottom of the refrigerated bag. Success at augmenting recipient platelet counts was limited both by volume constraints and because platelets, separated and stored in the cold, circulated poorly in recipients.<sup>6</sup> Mourad recognized that if PRP were allowed to come to room temperature before hard centrifugation, following supernatant plasma expression into another container, the platelet pellet could be gently resuspended in approximately 50 mL of residual plasma after a short resting period.<sup>7</sup> Building upon this finding, Murphy and Gardner revolutionized platelet therapy by demonstrating that platelets maintained at room temperature (20 to 24°C) throughout their entire ex-vivo interval were easily concentrated and circulated quite well in recipients.<sup>6</sup>

Since the early 1970s, room-temperature citrated whole blood has been transported from the collection site to a manufacturing facility and processed into Red Blood Cells (RBCs), plasma, and room-temperature PC components [Fig 12-1(A)]. Small changes to this process have been introduced over the years (eg, optimization of bag materials and centrifugation protocols, introduction of new anticoagulants, integration of leukocyte reduction filters into collection sets), but production of PCs from a PRP intermediate remains the only FDA-licensed method of WBD platelet production in the United States today. Room-temperature whole blood storage was initially limited to 4 hours after collection, but current guidelines allow an 8-hour period between venipuncture and platelet production.<sup>8,9</sup> There is increasing interest in extending this period for operational reasons. The overnight hold of whole blood donations eliminates the need for multiple transport runs from remote collection sites to the manufacturing facility and allows manufacturing on one rather than multiple shifts. Data exist to support the maintenance of PRP platelet quality after an overnight hold, but RBC quality and leukocyte reduction issues remain to be addressed.<sup>10,11</sup>

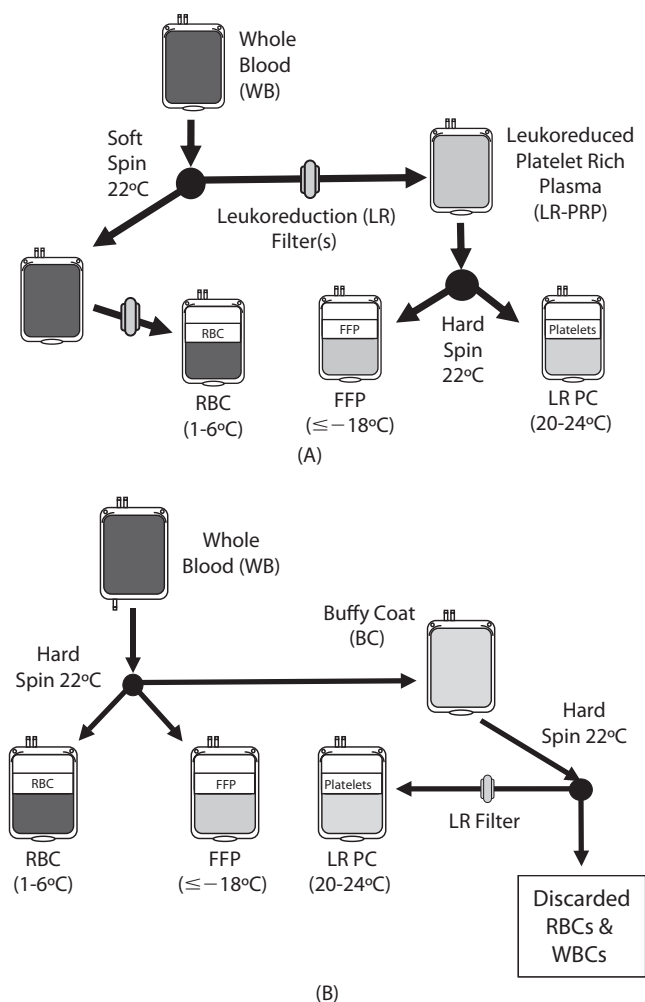


**Figure 12-1.** Platelet production methods.

RBC = Red Blood Cell; FFP = Fresh Frozen Plasma; LR = leukocyte reduced; PC = platelet concentrate.

Platelets derived from whole blood are known as Platelets (colloquially, random-donor platelets). AABB and the FDA require that a unit of Platelets contains at least  $0.55 \times 10^{11}$  platelets.<sup>12(p35-36)</sup> Modern concentrates actually contain an average of  $0.9 \times 10^{11}$  platelets with a relatively wide ( $\sim 0.27 \times 10^{11}$ ) standard deviation.<sup>1</sup> This requires a pool of at least five concentrates to ensure that, after a 6% to 10% loss during pooling, 99% of pools contain a dose of  $3 \times 10^{11}$ , the AABB/FDA-stipulated minimum content of Apheresis Platelets.<sup>12(p36)</sup> Prestorage leukocyte reduction is increasingly accomplished routinely during manufacture (Platelets, Leukocytes Reduced) with a loss of 4% to 5% of platelet content in the filter.<sup>13</sup> Leukocyte reduction results in a  $\geq 3$  log reduction in white cell (WBC) content to a level below AABB and FDA requirements for  $<0.83 \times 10^6$  WBCs per PC (one-sixth of the  $<5 \times 10^6$  threshold required for Apheresis Platelets).<sup>12(p30)</sup> PRP-PCs are suspended in autologous plasma anticoagulated with citrate-phosphate-dextrose (CPD), or CPD

with either twice the amount of dextrose (CP2D) or adenine (CPDA-1).

Platelets and Platelets, Leukocytes Reduced may be pooled into a licensed container specifically validated for prestorage pooling. This pool, produced by blood suppliers, has a 5-day outdate (midnight of the 5th day following collection). However, Platelets or Platelets, Leukocytes Reduced that are pooled after storage as individual concentrates outdate 4 hours after pooling.

## Preparation of Platelets from Whole Blood—the Buffy Coat Method

In Europe, concern about white cell content in RBC components and the need for plasma derivatives resulted in a different method of component production.<sup>14</sup> Initial hard centrifugation causes the density-dependent partition of plasma, a platelet- and white-cell-containing buffy coat (BC), and red cells. Both manual and automated devices exist to force plasma and red cells into different containers, leaving the BC in the primary container. Four to six BCs are combined, resuspended in plasma from a male donor (in the United Kingdom) or in a platelet additive solution (continental Europe) and soft centrifuged to remove red cells and white cells [Fig 12-1(B)]. The resultant product is passed through a leukocyte reduction filter to produce a prestorage pooled, leukocyte-reduced PC. This process results in lower initial white cell content, facilitating compliance with European standards for leukocyte reduction, which require  $<1 \times 10^6$  WBCs in the resultant pooled product.<sup>15</sup> Compared with the PRP manufacturing process, the BC process yields an average platelet product with a nearly equivalent platelet content. In addition, the RBC component has about 20 mL fewer red cells and 1 log fewer white cells. There is also about 30 to 75 mL more recovered plasma.<sup>16</sup> In 2004, the Canadian Blood Services initiated a switch from PRP to BC production because of perceived advantages, the most important of these being logistical. BC production employs butanediol plates within transport containers to rapidly cool and hold whole blood at 20 to 24°C for up to 24 hours before manufacturing. Newly available automated pooling technology contributes to process control, making BC production even more economical.<sup>17</sup>

In Europe, there is an approximately 50:50 split between the use of BC- and apheresis-derived PCs.<sup>14</sup> In Canada (Quebec excepted), 70% of platelets are derived from whole blood donation.<sup>18</sup> Denmark, Finland, and the Netherlands prepare 85% to 95% of their concentrates by the BC method.<sup>14,18</sup> These countries demonstrate that a national platelet supply can be derived almost entirely from collected whole blood rather than relying upon apheresis PC production.

In contrast to PRP-PCs centrifuged against the platelet container, BP-PCs are centrifuged against cellular elements of the whole blood unit. This may lead to less platelet activation during PC production relative to PRP-PCs.<sup>19</sup> Activation is one mechanism of platelet loss during storage.

## Preparation of Platelets by Plateletpheresis

Support of HLA-alloimmunized platelet-refractory patients requires platelets from a single donor. Apheresis-derived platelets are known as Apheresis Platelets (colloquially, single-donor platelets). In the mid-1970s, automated instruments were developed to replace high-volume, dedicated-donor PC collections (serial whole blood donations alternating with red cell and plasma reinfusion). As these cell separators became more widely available, inevitably, the economics of supporting plateletpheresis programs drove collections beyond these niche needs and apheresis-derived units were offered as a substitute for WBD units. Improved computer controls allowed apheresis technology to evolve beyond tedious cycles to continuous processing, which has significantly decreased procedure time and extracorporeal blood volumes. As the efficiency of apheresis separators improved and platelet yields increased well beyond the content of “standard” pools of six WBD-PCs, blood centers began to “split” apheresis units, providing therapeutic doses for two and sometimes three adults. In-process leukocyte reduction became possible with better separation controls, so Apheresis Platelets, Leukocytes Reduced now routinely contain  $<5 \times 10^6$  WBCs. Unlike WBD-PCs, red cell content in apheresis PCs is quite low in most units, often (but not always) below the dose of red cells known to produce RhD alloimmunization.<sup>20,21</sup>

The various apheresis instruments collect platelets in slightly different ways, leading to subtle differences in platelet populations and postprocedure activation. (See Chapter 41 for more detail.) Several variations on the earliest models employ a disposable plastic centrifuge chamber from which a layer of concentrated platelets is siphoned into a collection bag. More commonly employed instruments use a spinning channel with two-stage plastic inlays to initially separate the red cells from the PRP, followed by plasma removal before collection of the final platelet product. Yet another technology uses an elutriation mechanism to move larger platelets away from the red cell layer, separates the PRP, and then hyperconcentrates platelets along a collection bag wall before plasma resuspension in a storage container. This heterogeneity of collection technologies means that apheresis products are not necessarily similar nor will they always produce equivalent platelet increments in a given recipient.<sup>22</sup>

## Alternative Sources of Platelets

Although not currently cost-effective, efforts to generate platelets *ex vivo* from hematopoietic progenitor cells or cell lines may eventually be feasible. The equivalent of two WBD-PCs has been isolated from serial culture and *in-vitro* processing of a cord blood unit using various biological response modifiers.<sup>23</sup> These platelets appear morphologically and functionally normal *in vitro*. Development of this technology, however, faces a number of hurdles that make it unlikely to be implemented within the near future.

## Storage Conditions

Once the importance of room-temperature processing and storage was appreciated, platelets were kept for up to 3 days in the first generation of plastic containers. As improvements were made in platelet storage technology, shelf life was first increased to 5 days, and then to 7 days. In the mid-1980s the FDA mandated a return to 5-day shelf life because of increasing reports of bacterial sepsis associated with prolonged 20 to 24°C storage.<sup>24</sup> With the introduction of bacteria detection methods in 2004 (discussed in detail in Chapter 49), shelf life was again extended an additional 2 days for apheresis platelets screened using specific technologies.<sup>25</sup> Recurring concerns in early 2008 regarding bacterial contamination has yet again, resulted in a move away from 7-day platelet storage. Future improvements in bacteria detection technology, however, might for a third time, reverse the trend back to 7-day platelet storage.

Platelets survive for approximately 9 to 10 days in the circulation unless they are removed by senescence, activation associated with basal maintenance of vascular integrity (normally consuming platelets at a level of  $\sim 7.1 \times 10^9/\text{L/day}$ ) or used to correct pathologic bleeding.<sup>26</sup> Room-temperature storage appears to slow platelet metabolic activity by almost 60% compared with *in-vivo* activity at 37°C.<sup>27</sup> This suggests that 7 days of storage may lead to only 3 days' worth of *in-vivo* senescent loss. Storage conditions that minimize platelet loss by inhibiting platelet activation, apoptosis, or metabolic exhaustion might, in theory, permit the extension of PC shelf life well beyond 7 days. The critical variables affecting stored platelet health are: 1) temperature, 2) metabolic fuel availability, and 3) respiratory capacity. The latter is dependent on platelet content, gas diffusion through the plastic container, and agitation. Activation occurring during collection and processing appears to be mostly reversible, but excessive activation may also lead to platelet loss.

## Metabolic Concerns During Storage—Temperature

When platelets are exposed to the cold, their plasma membranes undergo phase transitions that bring lipid rafts together, clustering activating phosphoinositides and exposing neoepitopes.<sup>28–30</sup> This *in-vivo* platelet cold-sensitivity presumably facilitates activation at wound sites on the cooler body surface, while resulting in the removal of repetitively primed platelets to prevent core thrombosis.<sup>29,30</sup>

When platelets are stored in the cold, time- and temperature-dependent elevation of intracellular calcium results in sphering of normally discoid platelets as calcium-dependent gelsolin activation cleaves actin cytoskeletal elements.<sup>29,31</sup> Reversibility of this initial shape change after brief cold exposure is dependent upon the integrity of the circumferential platelet microtubular array, which is also

cold-sensitive.<sup>32</sup> Membrane phase transitions at temperatures below 30°C bring phosphoinositide-rich lipid rafts together.<sup>29</sup> These phosphoinositides promote actin polymerization, resulting in spiny projections on platelets stored in the cold.<sup>29,33</sup> Spherical and dendritic shape changes are early manifestations of agonist-induced platelet activation and, presumably, prime cold-exposed platelets for activation. Cold-exposed platelets are still able to function normally despite morphologic changes, although such changes correlate negatively with the ability of transfused platelets to circulate.<sup>6,34-36</sup> More recent investigations may explain this apparent paradox. Repeated or prolonged hypothermic platelet priming results in clustering of the principal von Willebrand factor receptor, glycoprotein (GP) Ib/IX/V.<sup>30</sup> Function of the receptor, however, is not affected by clustering. Lectin-binding domains of hepatic macrophage  $\alpha_M\beta_2$  receptors recognize clustered GPIb $\alpha$  subunits and remove these platelets. Efforts to block in-vivo platelet destruction by glycosylating newly exposed  $\beta$ -N-acetylglucosamine residues on clustered GPIb $\alpha$  molecules have thus far been successful on murine, but not human platelets.<sup>37,38</sup>

Not unexpectedly, the reversibility of cold-induced platelet injury is both time- and temperature-dependent. Figure 12-2 shows the effect of 18 hours of storage at various temperatures upon the half-life of radiolabeled autologous platelets after reinfusion into healthy volunteers. Temperatures above 20°C resulted in normal platelet survival.<sup>6</sup> Holme et al<sup>41</sup> reported that significant loss of predicted platelet viability occurred after 24 hours at 18°C, 16 hours at 16°C, 10 hours for 12°C and 6 hours at 4°C. Storage above the 20 to 24°C temperature described as optimal by Murphy and Gardner did not result in measurable damage, but was accompanied by increased metabolism compared with room temperature storage. The salutary metabolic effects of room temperature storage include decreased in-vitro aging and slower accumulation of toxic metabolites within the platelet container.

## Metabolic Concerns During Storage—Metabolic Fuel Availability

Stored platelets must catabolize substances within their suspension medium to regenerate adenosine triphosphate (ATP), the principal “energy currency” of the cell spent in the maintenance of cellular integrity. Figure 12-3 shows the pathways for ATP regeneration.

Seminal studies of glucose consumption and lactate production in PRP-PCs revealed a 1:2 ratio consistent with predominantly anaerobic metabolism of glucose.<sup>40</sup> Pyruvate dehydrogenase, which produces acetic acid for the aerobic citric acid cycle, appears to be downregulated in stored platelets. Glucose metabolism in platelets ends with the production of only 2 moles of ATP per mole of glucose consumed. Lactic acid is buffered primarily by plasma bicarbonate, which results in the production of CO<sub>2</sub>.<sup>41</sup> This CO<sub>2</sub>, a volatile acid, diffuses through

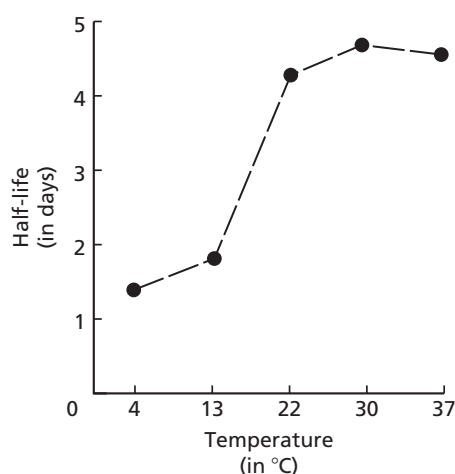


**Figure 12-2.** In-vivo survival of radiolabeled autologous platelets stored at various temperatures for 18 hours.

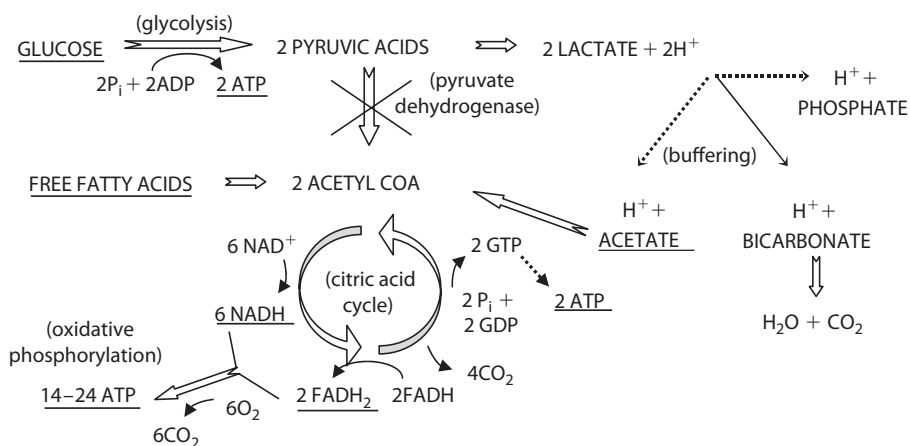


**Figure 12-3.** Pathways of platelet adenosine triphosphate (ATP) regeneration from inorganic phosphate (P<sub>i</sub>) and adenosine diphosphate (ADP). Metabolic fuels are underlined. Energetic intermediates are underlined. Carbon dioxide (CO<sub>2</sub>) is produced from hydrogen ion (H<sup>+</sup>) buffering and oxidative metabolism of acetyl CoA. GDP/GTP = guanosine di-/triphosphate; NAD/NADH = nicotinamide adenine dinucleotide/reduced NAD; FADH/FADH<sub>2</sub> = reduced flavin adenine dinucleotide forms.

the storage container wall. Progressive depletion of bicarbonate thus occurs through lactic acid buffering as well as slow spontaneous dissociation to  $\text{CO}_2$  and water. Once bicarbonate is consumed, continued lactic acid production ultimately results in a deleterious decrease in product pH.

Aerobic metabolism through the citric acid cycle and oxidative phosphorylation is a far more efficient source of energy, yielding 8 to 9 moles of ATP and 2 moles of  $\text{CO}_2$  per mole of acetic acid entering the cycle. Comparison of platelet oxygen consumption (6 moles of ATP produced per mole of  $\text{O}_2$  consumed) with lactate production (1 mole of ATP per mole of lactate) suggests that approximately 85% of platelet energy is derived from aerobic metabolism.<sup>40</sup> Studies using radiolabeled palmitate point to free fatty acids derived from plasma triglycerides as the predominant metabolic fuel for platelets stored in plasma.<sup>42</sup> A small, rather insignificant component results from metabolism of the amino acid glutamine.<sup>43</sup> Efforts to identify plasma-sparing platelet additive solutions (PAS) led to the discovery that acetate could serve as an effective alternative fuel to fatty acids.<sup>44</sup> As described below, acetate also provides a valuable lactate-buffering function through consumption of a hydrogen ion during its metabolism.

Under low oxygen conditions, glycolysis is upregulated six- to sevenfold, the so-called Pasteur effect.<sup>45</sup> Although this effect satisfies energy requirements in the absence of aerobic metabolism, it results in a considerable increase in lactic acid production, which can exacerbate plasma buffer exhaustion and produce deleterious declines in pH. Mechanical factors also affect platelet metabolism. Mitochondrial oxygen starvation can occur with the interruption of agitation during excessively long periods of shipping, accompanied by an expected increase in lactate generation and pH decrements.<sup>46</sup> Studies have shown that platelets begin to suffer deleterious effects below a pH of 6.5 to 6.8 at room temperature (22°C) and are irreversibly damaged in the face of a sustained pH below 6.2 at 22°C.<sup>41,47</sup> Efforts to extend platelet storage have been limited by the relatively constant glycolytic production of lactate, even with maintenance of adequate oxygenation. The availability of suitable lactic acid buffers may not be the only metabolic challenge. Attempts to develop glucose-free additive solutions to minimize the production of lactate have not been successful. Despite the presence of sufficient amounts of alternate fuels (such as acetate), once PCs exhaust their supply of glucose, they increase their metabolic rate and deplete their adenine nucleotides, leading to cell death.<sup>44</sup> The reasons for this are poorly understood.

Adenine nucleotide depletion during storage has been characterized in PRP-PCs.<sup>48</sup> ATP and adenosine diphosphate (ADP) are present in platelets in both metabolic and storage pools. The former provides for the metabolic needs of the platelet (ATP:ADP ratio 8:1) and the latter, present in delta granules and released upon platelet activation, serve as autocrine/paracrine second messengers (ATP:ADP ratio 2:3). Both pools were noted to decline equally throughout storage in experiments employing radiolabeled adenine, suggesting some element of platelet activation or lysis in addition to metabolic turnover. After 7 days of

storage, adenine nucleotide levels in PCs decline to 70% of those initially present.<sup>49</sup> Supplementation of the anticoagulant with adenine (CPDA-1 instead of CPD) resulted in better ATP maintenance throughout storage. Unlike red cells, however, there is no evidence that adenine-containing media preserve platelet function better than those without adenine.

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## Metabolic Concerns During Storage—Respiratory Capacity

Maintenance of aerobic metabolism requires a  $\text{PO}_2$  above 5 to 10 mmHg.<sup>40</sup> Because oxygen consumption is relatively constant per platelet, presence of a high total platelet bag content stresses the oxygen permeability of the storage container. When PC consumption exceeds the ability of the container to transport oxygen, the  $\text{PO}_2$  will decline to hypoxic levels over several days, leading to upregulation of glycolysis, lactic acid accumulation, and a deleterious decrease in pH. Gases diffuse across storage container walls driven by their gradients once the plastic is saturated. Each manufacturer's container has a different gas permeability related to wall thickness and the plastic's unique gas transport capacity. Oxygen delivery also depends upon the container's ratio of volume-to-surface area as well as continuous agitation, which promotes gas diffusion and helps maintain normal platelet oxygen consumption.

Kilkson et al<sup>40</sup> demonstrated that across the  $\text{CO}_2$  concentrations seen in PCs stored in the least permeable containers (10 to 100 mmHg), little pH change is attributable to formation of carbonic acid from aqueous  $\text{CO}_2$  and  $\text{H}_2\text{O}$ . Lactate concentration was shown to be the principal determinant of pH. Thus, high  $\text{CO}_2$  permeability is not as critical as is good oxygen permeability. High  $\text{CO}_2$  permeability, in fact, can lead to an early increase in pH as the  $\text{PCO}_2$  falls from physiologic levels, unbalanced by significant lactic acid accumulation. A pH >7.4 at 22°C may result, which has been associated with cellular damage with earlier forms of agitation. Although European regulators have set 7.4 pH as the upper limit of acceptable end-storage at 22°C, a recent study noted that using modern forms of agitation, a pH >6.3 at 22°C had no predictive value for platelet viability (in PCs compliant with licensed maximum and minimum platelet contents and concentrations).<sup>50</sup>

From the foregoing discussion of metabolic fuel requirements, it is evident that platelet storage is limited by the ability of suspension medium buffers to handle constitutive glycolytic lactate production. High platelet content increases lactate production rates even further, because high platelet concentrations result in fewer plasma buffers per respiring platelet. Less evident is the deleterious effect of low platelet contents and concentrations. Adverse effects in these situations result from low  $\text{CO}_2$  and lactate production, which initially result in an increase in PC pH. Why these products develop metabolic exhaustion and adenine nucleotide depletion is not entirely clear, but some forms of agitation further increase this effect at higher pH values.



The very first containers, made of polyvinylchloride (PVC) plasticized with di-(2-ethylhexyl) phthalate (DEHP) were originally designed for freezing plasma. Serendipitously, they allowed enough oxygen and CO<sub>2</sub> diffusion across their walls to permit room temperature platelet storage of relatively lower content PCs for 3 days before the pH decreased to unacceptable levels. Containers were later specifically designed for improved gas permeability as trial and error revealed that PCs stored in more gas-permeable containers had lower rates of pH failure (pH <6.2 at 22°C).<sup>51</sup> So-called second-generation containers appear to be able to store PRP-PCs for at least 7 days without deleterious pH decrements. Because apheresis PCs and prestorage pooled PRP-PCs have such heterogeneous contents, each unit must be assayed to ensure that total platelet content and concentration limits validated by the container manufacturer are not exceeded. The platelet concentration of donor blood limits individual WBD-PC content to values below those accommodated by newer storage containers. Second-generation containers currently in use are manufactured from polyolefin, or PVC that is thinner or plasticized with different compounds such as triethyl hexyl trimellitate and butyryl-tri-hexyl citrate.<sup>52</sup> These bags provide nearly twice the oxygen permeability of first-generation DEHP-plasticized PVC containers.<sup>53</sup>

The need for continuous agitation was also recognized as a means of facilitating oxygen utilization.<sup>51,54</sup> Several different types of platelet rotators have been used over the years. Ferris wheel and elliptical rotators have given way to face-over-face tumblers (3-6 rpm) and flatbed platform shakers (50-70 cpm) because of inefficient maintenance of pH or damage to platelets at pH >7.4 at 22°C.<sup>41</sup> Initially thought to promote the diffusion of oxygen throughout the PC, agitation appears to exert a more complex effect on platelet metabolism. Several groups have shown that PO<sub>2</sub> was maintained in unagitated PCs and that mixing of products even once a day could prevent injurious pH decrements in relatively lower content platelet products.<sup>55,56</sup> Agitation, therefore, may also facilitate oxygen utilization by platelet mitochondria or prevent settling and a platelet contact-mediated switch from oxidative to glycolytic metabolism.<sup>57</sup> In any case, even with relatively higher content PCs, agitation may be safely interrupted for 24 hours (as during shipping) without undue lactate accumulation and subsequent development of injuriously low pHs.<sup>57,58</sup>

## The Platelet Storage Lesion

Stored platelets experience a progressive decline in function accompanied by characteristic morphologic changes. Studies document up to 20% loss of platelet recovery through 5 days of storage.<sup>59</sup> In containers currently licensed in the United States, Day 7 recovery is approximately 83% of Day 5 recovery.<sup>60,61</sup> This progressive damage has been termed the platelet storage lesion.<sup>62</sup> Well-characterized changes are seen in common tests assessing platelet morphology, activation, cell metabolism/function, and senescence (apoptosis). Some, but not all, of these changes are reversible upon platelet transfusion.<sup>63</sup>

The gold standard for evaluating product efficacy has been adjusted count increments after patient transfusion, ideally accompanied by bleeding time studies demonstrating improved hemostasis. In practice, however, such studies are not often performed because count increments and bleeding times are affected by uncontrollable patient variables, thus requiring very large studies to document the impact of storage condition changes on the platelet storage lesion. An acceptable alternative involves the reinfusion of healthy volunteers with autologous stored platelets radiolabeled with chromium-51 or indium-111. (Cell kinetic studies are described in greater detail in Chapter 21.) Percent recovery and survival may be calculated by serial assay of postinjection blood radioactivity. Platelet recovery is significantly affected by individual differences in splenic sequestration (usually ~33%) and blood volume estimation errors during calculations. In an effort to standardize interpretation, Murphy proposed that products be evaluated against concurrently injected fresh autologous platelets to establish a fixed standard.<sup>64</sup> This will facilitate comparison of interventions designed to ameliorate the storage lesion. Although in-vitro tests have attempted to predict rapidly and economically the ability of transfused platelets to circulate, only extreme values have documented any correlation with outcomes.<sup>65</sup> Accordingly, batteries of tests are often examined to ensure product equivalency after minor changes, or as the first step in assessment of more substantive changes in storage methods.

Alterations from normal discoid morphology are observed during platelet storage. Kunicki described a scoring system that assigned 4 points to discs, 2 to spheres, 1 to dendritic forms, and none to ballooned platelets.<sup>66</sup> Multiplied by the percentage of each form seen under phase contrast microscopy, scores decline from a maximum of 400 as changes associated with the storage lesion progress. Others have advocated simply reporting the percentage of discoid forms. The assay of the extent of shape change uses aggregometry to measure the decrease in light transmission after ADP stimulation of stirred PRP in the presence of EDTA to prevent aggregation.<sup>67</sup> Because only discoid platelets can change shape and block more light, the assay provides a more objective measure of the percentage of discoid platelets. All three approaches appear to correlate somewhat with in-vivo recovery and survival at high or low assay values, but predict less well for in-between values. Mean platelet volume decreases throughout storage when membrane loss from dendritic forms occurs or when activation or apoptosis result in microvesiculation.<sup>68</sup>

Platelet activation during processing and throughout storage is accompanied by surface expression of sequestered granular membrane proteins (P-selectin and CD63) and conformational changes of the fibrinogen receptor, GP-IIb/IIIa. Release of specific granular contents into the supernatant (β-thromboglobulin, platelet factor 4, etc) also occurs throughout platelet storage as a manifestation of activation. This may not be specific for activation, however, because granular content release also occurs with platelet lysis. Similarly, platelet procoagulant activity (surface expression of membrane phosphatidylserine), appears not only with activation, but also during cellular apoptosis. None of these indicators of platelet

activation appear to correlate reliably with platelet recovery and survival. Studies of storage medium supplementation with activation inhibitors, however, suggest that activation contributes in part to the platelet storage lesion.<sup>69</sup> Use of activation inhibitors in storage media has not gained widespread acceptance because of inconsistent results, toxicity, and the expense of licensing novel storage systems.

The ability of stored platelets to function normally has been assessed in several ways, only one functional assay of which—hypotonic shock response—has proven to be of value in assessing platelet viability. Measurements of platelet ATP levels do not consistently provide reliable information.<sup>49</sup> Aggregation studies similarly fail to predict platelet viability. Responsiveness to single agonists rapidly declines during storage, but can recover with plasma rescue or in-vivo reinfusion.<sup>63</sup> Responses to agonist mixtures, while better preserved, are still not helpful in assessing viability.<sup>70</sup> The hypotonic shock response or osmotic reversal reaction exposes platelets to a hypotonic environment. Metabolically active platelets rapidly extrude water after initially swelling. Recovery of normal intracellular water content can be quantified using light transmittance. Significant changes in values for this assay have been shown to correlate with in-vivo recovery and survival.<sup>67</sup>

Markers associated with cellular apoptosis accumulate throughout platelet storage with evidence of caspase activation, structural protein degradation, and changes in mitochondrial membrane potential.<sup>71,72</sup> Apoptotic inhibitors, while ameliorating some of these changes, have not resulted in improvements in platelet viability, casting doubt upon cellular senescence as a significant contributor to the platelet storage lesion.

## Platelet Additive Solutions (Synthetic Storage Solutions)

In the United States, all platelets are stored in plasma. In Europe, synthetic storage solutions, also called platelet additive solutions,

have been used routinely for at least a decade.<sup>73</sup> The use of PAS allows: 1) greater plasma recovery from whole blood donations for transfusion or fractionation, 2) a minimizing of the adverse effects mediated by plasma (eg, allergic reactions), 3) use of photochemical pathogen reduction technologies, because the presence of plasma may interfere with the technology system, and 4) potential improvements in platelet storage through engineered manipulation of the storage medium.<sup>74,75</sup> It is likely that some PAS will be licensed and made available for use in the United States in the near future, considering the mandate to reduce the level of plasma in platelet concentrates as a strategy to mitigate transfusion-related acute lung injury.<sup>76</sup>

Early studies demonstrated the feasibility of suspending PRP-PCs in a commercially available neutral intravenous crystalloid solution, Plasmalyte A (composition listed in Table 12-1).<sup>77</sup> The 10% to 20% plasma carried over in the storage medium provided the sole source of glucose and bicarbonate. In-vitro results were quite encouraging but although previous versions of this PAS containing additional glucose and citrate were shown to be promising in vivo, the solution was not successfully commercialized.<sup>78</sup> When Murphy tested another balanced salt solution, the in-vivo results were disappointing.<sup>79</sup> In retrospect, the deletion of acetate from Murphy's so-called PSM-2 solution was likely responsible for the pH failures seen in-vitro. The poor in-vivo recoveries of a phosphate-buffered version of this PAS may have been caused by glucose exhaustion due to the absence of acetate, despite continued free fatty acid availability. Acetate can efficiently substitute in the citric acid cycle for free fatty acids, the presumed primary substrate for oxidative metabolism (Fig 12-3), decreasing both the glycolytic rate and lactic acid generation.<sup>80</sup> Acetate must also be transformed to acetic acid to enter the cycle, removing hydrogen ions produced by the anaerobic metabolism of glucose. This bicarbonate-sparing buffering effect also helps preserve pH. From Murphy's experience and other studies, it appears likely that some glucose must be available throughout storage. Holme

**Table 12-1.** Composition of Selected Platelet Additive Solutions

| Element (in mM)    | PlasmaLyte-A<br>(Normosol-R,<br>Hospira) | PAS-II<br>(T-Sol, Baxter) | PAS-III<br>(InterSol, Baxter) | Composol<br>(Composol,<br>Fresenius) | PAS-IIIM<br>(SSP+,<br>Macopharma) | GASP-BIC<br>(PAS-G,<br>Pall) |
|--------------------|------------------------------------------|---------------------------|-------------------------------|--------------------------------------|-----------------------------------|------------------------------|
| Sodium chloride    | 90                                       | 115.5                     | 77.3                          | 90.0                                 | 69.3                              | 110.0                        |
| Potassium chloride | 5                                        | —                         | —                             | 5.0                                  | 5.0                               | 5.0                          |
| Magnesium chloride | 3                                        | —                         | —                             | 1.5                                  | 1.5                               | 3.0                          |
| Citric acid        | —                                        | —                         | —                             | —                                    | —                                 | 7.5                          |
| Na citrate         | —                                        | 10.0                      | 10.8                          | 11.0                                 | 10.8                              | —                            |
| Na acetate         | 27                                       | 30.0                      | 32.5                          | 27.0                                 | 32.5                              | 15.0                         |
| Na bicarbonate     | —                                        | —                         | —                             | —                                    | —                                 | 26.4                         |
| Na gluconate       | 23                                       | —                         | —                             | 23.0                                 | —                                 | —                            |
| Na phosphate       | —                                        | —                         | 28.2                          | —                                    | 28.2                              | 4.0                          |
| Glucose            | —                                        | —                         | —                             | —                                    | —                                 | 30.0                         |

and Gullikssen both noted the association of glucose exhaustion (even in the presence of alternate fuels) with adenine nucleotide depletion and loss of platelet viability.<sup>44</sup> The addition of glucose to PAS, however, is technically difficult because glucose caramelizes upon heat sterilization at the neutral or slightly basic PAS pHs best suited to maintain PC pH. Accordingly, most commercially available PAS do not contain additional glucose and require 20% to 40% plasma carryover. One PAS adds glucose to a low pH additive solution in combination with bicarbonate tablets in the storage container to re-adjust the storage pH.<sup>81</sup> Table 12-1 lists the components of commonly used PAS.

In addition to sparing bicarbonate, PAS contents have been designed to modulate the glycolytic rate and downregulate platelet activation. Citrate, important in maintaining an anticoagulant environment, upregulates glycolysis and renders platelets more susceptible to activating stimuli. Consequently, use of the lowest possible concentrations of citrate in the medium appear desirable.<sup>44</sup> Phosphate similarly has both salutary and detrimental effects in PAS.<sup>82</sup> Phosphate serves as a buffer and in apheresis platelets suspended in acid-citrate-dextrose (ACD) instead of the CPD/CP2D used in PRP-PCs and BC-PCs, is important in maintaining adenine nucleotide levels. It also upregulates glycolysis and consequently mandates more plasma carryover or addition of glucose to PAS. Magnesium and potassium both appear to decrease platelet activation and may downregulate glycolysis as well.<sup>44</sup> Studies with a number of platelet activation inhibitors (eg, prostaglandin E1, theophylline) suggest that these substances may enhance PAS efficacy, but safety concerns related to routine transfusion in varied patient populations significantly hinders commercial applicability.<sup>83</sup>

Robust clinical studies comparing platelets stored in plasma with the many PAS under investigation, are currently lacking. In studies to date examining corrected increments in patients, there appears to be somewhat better maintenance of platelet viability in 100% plasma early in storage, an effect that gradually disappears by Day 5.<sup>84</sup> Allergic reactions were markedly reduced with PAS platelets in several studies.<sup>84</sup> Although in-vitro data support slight advantages to PAS IIIM (Baxter S.A., Lachâtre, France) and Composol (Fresenius Kabi, Bad Homburg, Germany) over other PAS, the clinical arm of this comparison has not yet been carried out. Although there is much research still to be performed, PAS remains as an important future tool for reducing the concurrent infusion of plasma along with therapeutic doses of platelets.

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## Component Modification

Several other PC modifications are occasionally required for various subsets of patients. Cellular products containing viable lymphocytes must be irradiated before transfusion to patients who are susceptible to graft-vs-host disease. An irradiation dose of 25 Gy (15 to 50 Gy) has no significant effect on common in-vitro parameters even after 7 days of storage following irradiation as early as Day 1.<sup>85</sup> Similarly, little or no effect of irradiation was seen on in-vitro storage parameters or in-vivo recovery and

survival after 5 days of storage of platelets irradiated with up to 30 Gy on Days 1 or 3.<sup>86,87</sup>

Patients with moderate to severe allergic reactions to plasma components or newborns with neonatal alloimmune thrombocytopenia receiving maternal platelets benefit from plasma removal by platelet washing. Some institutions also wash platelets to remove ABO-incompatible plasma.<sup>88</sup> Washing, however, leads to significant loss of platelets as well as a decrement in platelet function while also reducing the product shelf life to 4 hours. A standard saline wash procedure using the COBE 2991 instrument (CaridianBCT, Lakewood, CO) has been described.<sup>89</sup> Platelet losses range from 8% to 29%.<sup>90,91</sup> A study examining the effect of 0.9% saline or a 12.6% ACD-buffered saline wash fluid compared with unwashed platelets showed a 68% reduction in in-vivo recovery for saline-washed platelets and 28% reduction in recovery with ACD-buffered saline.<sup>92</sup> Thus, actual and functional losses may represent up to two-thirds of the pre-wash platelet content. For IgA-deficient patients, saline alone may not remove as much IgA as citrate-buffered saline because of the low isoelectric point of IgA and its coprecipitation with platelets in an acidic saline wash fluid.<sup>93</sup>

For transfusions in utero or for volume-sensitive neonates, additional concentration of platelet concentrates can be achieved by further volume reduction via plasma removal. This technique has also been used to minimize infusion of maternal antibodies or ABO-incompatible plasma, although washing removes more antibody. Procedures have been described for volume reduction that results in <15% loss of platelets and relatively preserved in-vivo viability.<sup>94</sup> PCs are centrifuged and excess plasma expressed from the unit followed by gentle resuspension by manual means or by use of an agitator. Volume-reduced PCs maintain acceptable in-vitro properties in plastic syringes at room temperature or in neonatal incubators (at 37°C).<sup>95</sup> PCs should be transfused as soon as possible after volume reduction in a closed system, employing sterile connection devices, and must be used within 4 hours if prepared in an open system, such as via spike entry.

Most apheresis platelets are leukocyte reduced during the separation process. Leukocyte reduction of WBD-PCs may be accomplished through bedside filtration or by using a more quality-controlled prestorage filtration process. An important benefit of pre- rather than poststorage filtration is the early removal of white cells that would otherwise produce cytokines throughout storage. Prestorage leukocyte reduction has resulted in a 75% to 90% reduction in febrile, nonhemolytic reactions after platelet transfusion.<sup>96,97</sup> Leukocyte reduction also reduces cytomegalovirus transmission and platelet alloimmunization.<sup>98,99</sup>

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## Novel Storage Techniques

The short shelf life and significant infrastructure needed to maintain room-temperature, continuously agitated, liquid PCs have encouraged exploration of alternative storage approaches. Cold liquid storage clearly minimizes the impact of bacterial contamination and also promises to extend shelf life by further

slowing platelet metabolism. Unfortunately, while the function of refrigerated platelets is normal, they circulate poorly and despite success in mice, galactosylated clusters of GPIb $\alpha$  subunits still result in rapid reticuloendothelial clearance in humans.<sup>34-38</sup> Additional work to overcome the recognition of these clusters by hepatic  $\alpha_M\beta_2$  receptors is required. One group has reported stabilization of refrigerated platelet membranes with antifreeze glycoproteins derived from polar fish, but the safety and in-vivo activity of these platelets has not been described.<sup>100</sup>

Cryopreserved platelets can be prepared and stored for up to 10 years, but cost, inconvenience, procedure-related platelet loss, and functional decrements remain barriers to wider implementation. The most common method of freezing employs 5% to 6% dimethylsulfoxide (DMSO) as a cryoprotectant, and requires  $<-80^\circ\text{C}$  storage and postthaw washing to remove the DMSO.<sup>101,102</sup> The use of cryopreserved platelets results in corrected count increments that are 40% to 65% of the increments seen with liquid apheresis platelets transfused relatively early after storage.<sup>102,103</sup> The 50% to 66% loss of function in addition to the 15% to 20% platelet loss from transfers and washing may be mitigated by use of a commercial solution containing amiloride, adenosine, and sodium nitroprusside, which “stabilizes” platelets and allows reduction to a 2% DMSO content.<sup>104,105</sup> Studies have noted the superiority of controlled rate freezing over uncontrolled temperature reduction using both DMSO concentrations.<sup>106,107</sup> Frozen platelets are useful primarily for broadly alloimmunized patients who are refractory to allogeneic transfusion. Collected between successive rounds of high-dose chemotherapy, these units can support patients through short periods of severe thrombocytopenia.

Lyophilized platelets are attractive for use in forward-deployed military facilities where platelets are often unavailable. Animal studies in the 1950s showed no hemostatic effect of early preparations.<sup>108,109</sup> Bode and Read described a new technique using 1.8% paraformaldehyde to gently fix washed platelets, followed by freezing in 5% bovine albumin and lyophilization.<sup>110</sup> This preparation has been stored at  $-80^\circ\text{C}$  but may be stable at room temperature for shorter periods. Paraformaldehyde treatment appears to confer an additional benefit by killing bacteria and many viral pathogens. In-vitro studies show that these platelets, although reduced in surface glycoprotein content, are morphologically normal, support coagulation, and can be activated. In animal models, they participate in aggregates with normal platelets, shorten the bleeding time, and do not appear to induce disseminated coagulation.<sup>111</sup> In-vivo studies suggest, however, that the duration of action may be short following rehydration. Clinical studies in humans will be needed to define the product’s safety and efficacy, primarily in treatment of bleeding, rather than its prevention. An alternate method of lyophilization using trehalose instead of paraformaldehyde is still in preclinical testing.<sup>112</sup>

The role of platelet substitutes including platelet microvesicles, liposome-based hemostatic agents, fibrinogen-coated albumin microspheres, and red cells coated with fibrinogen or RGD

peptides is beyond the scope of this chapter and readers are referred to published reviews for additional information.<sup>111,113,114</sup>

## Acknowledgment

Scott Murphy, a platelet storage pioneer, authored this chapter for the three previous editions of this book. Platelet transfusion therapy originated from his seminal work. Scott’s intellectual curiosity, wit, and wisdom are sincerely missed. His spirit and words are woven into the fabric of this chapter.

## Disclaimer

The author has disclosed a financial relationship with Fenwal, Inc.

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# 13 Thrombocytopenia and Platelet Transfusion

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Thrombocytopenia is caused by disorders impairing platelet production, causing platelet destruction or leading to platelet sequestration. In addition, there are both congenital and acquired disorders of platelet function. These conditions may lead to the functional equivalent of thrombocytopenia, often despite the presence of a normal platelet count. Platelet transfusion represents an important therapeutic option for most of these disorders. However, under certain circumstances platelet transfusion is contraindicated. Therefore, correct diagnosis of the underlying etiology of thrombocytopenia is important.

Products for platelet transfusion include platelet pools derived from whole blood donation from multiple donors and apheresis units from individual donors. These components may be further manipulated depending on the requirements of the recipient. Allergic reactions to platelets can often be addressed by the administration of appropriate medications before transfusion. Platelet refractoriness represents a significant problem that develops in some patients who require repeated infusions of platelet products. Several therapeutic options exist to address platelet refractoriness, depending on the severity of the situation. These include the transfusion of crossmatched or HLA-matched platelets, the continuous infusion of platelets, and the administration of immune globulin, antifibrinolytic agents, or recombinant Factor VIIa. The development of platelet substitutes and the administration of thrombopoiesis-stimulating agents represent alternatives to platelet transfusion that are in various stages of clinical development and may ultimately reduce the need for donor-derived platelets.

## Thrombocytopenia

Thrombocytopenia refers to any reduction in platelet number below the lower limit of the normal range, which is about 150,000/ $\mu\text{L}$  in most laboratories. In the absence of other factors, however, the risk of bleeding is considered to be relatively modest until the

platelet count is less than 50,000/ $\mu\text{L}$ , and is not considered to be severe until the platelet count is less than 25,000/ $\mu\text{L}$ .<sup>1</sup> However, congenital or acquired abnormalities in platelet function may be associated with bleeding even when the platelet count is in the normal range.<sup>2,3</sup> A systematic diagnostic approach facilitates the correct diagnosis and appropriate management of thrombocytopenia and platelet dysfunction. Careful assessment of the medical history is imperative. In particular, review of concomitant medications is often revealing. The peripheral blood smear should be reviewed for morphologic abnormalities in any of the three lineages (myeloid, erythroid, megakaryocytic), which may provide important insight in to the underlying diagnosis, be it congenital or acquired. An overview of management options is presented in Table 13-1.

## Impaired Platelet Production

Disorders of platelet production may be congenital or acquired. Congenital disorders are relatively rare, whereas acquired disorders are more commonly encountered in clinical practice. In the absence of an apparent etiology, distinguishing decreased platelet production from increased platelet destruction or from splenic sequestration can sometimes be challenging. Decreased platelet production is generally characterized by normal platelet size on the peripheral blood smear and a normal mean platelet volume (MPV). In addition, the reticulated platelet count as measured by flow cytometry is low.<sup>4</sup> Although additional laboratory testing such as serology for platelet antibodies can be useful in specific instances, a marrow aspirate and biopsy may be required in order to determine the underlying disorder associated with thrombocytopenia.

Hematologic and nonhematologic malignancies are associated with replacement of the marrow space (myelophthisis). Alternatively, the marrow may be replaced with fibrosis. In general, these conditions usually present with pancytopenia. However, sometimes reduction in the platelet count is the first indication of these abnormalities. Platelet transfusions may be used appropriately for supportive care as needed while the underlying disorder is addressed.

## Platelet Sequestration

About one-third of the platelet mass is normally sequestered in the spleen.<sup>5</sup> Splenomegaly from any cause tends to increase



**Table 13-1.** Management Options for Disorders Affecting Platelets

| Disorder                               | Therapeutic Options                                                                                                                                                                                                           |
|----------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Impaired platelet production           | <ul style="list-style-type: none"> <li>• Platelet transfusion</li> </ul>                                                                                                                                                      |
| Splenic sequestration                  | <ul style="list-style-type: none"> <li>• Platelet transfusion</li> </ul>                                                                                                                                                      |
| Glanzmann's thrombasthenia             | <ul style="list-style-type: none"> <li>• Correction of any associated coagulopathy</li> <li>• Epsilon aminocaproic acid</li> <li>• Desmopressin</li> <li>• Platelet transfusion</li> <li>• Recombinant Factor VIIa</li> </ul> |
| Bernard-Soulier syndrome               | <ul style="list-style-type: none"> <li>• Platelet transfusion</li> </ul>                                                                                                                                                      |
| Type 2B von Willebrand disease         | <ul style="list-style-type: none"> <li>• Transfusion with intermediate purity Factor VIII concentrate</li> </ul>                                                                                                              |
| Platelet granule defects               | <ul style="list-style-type: none"> <li>• Epsilon-aminocaproic acid</li> <li>• Desmopressin</li> <li>• Platelet transfusion</li> </ul>                                                                                         |
| Immune thrombocytopenic purpura        | <ul style="list-style-type: none"> <li>• Prednisone or dexamethasone</li> <li>• Immune globulin</li> <li>• Platelet transfusion</li> </ul>                                                                                    |
| Thrombotic thrombocytopenic purpura    | <ul style="list-style-type: none"> <li>• Plasma exchange</li> <li>• Plasma infusion if plasma exchange is not immediately available</li> </ul>                                                                                |
| Hemolytic uremic syndrome              | <ul style="list-style-type: none"> <li>• Supportive care</li> <li>• Discontinuation of any offending medication</li> <li>• Consider plasma exchange in adults</li> </ul>                                                      |
| Disseminated intravascular coagulation | <ul style="list-style-type: none"> <li>• Treat underlying cause</li> <li>• Transfusion of blood products, including platelets, as required</li> </ul>                                                                         |
| Uremia                                 | <ul style="list-style-type: none"> <li>• Desmopressin</li> <li>• Conjugated estrogens</li> <li>• Platelet transfusion</li> </ul>                                                                                              |
| Heparin-induced thrombocytopenia       | <ul style="list-style-type: none"> <li>• Argatroban or lepirudin</li> </ul>                                                                                                                                                   |
| Posttransfusion purpura                | <ul style="list-style-type: none"> <li>• Immune globulin</li> <li>• Plasmapheresis</li> </ul>                                                                                                                                 |
| Neonatal alloimmune thrombocytopenia   | <ul style="list-style-type: none"> <li>• Immune globulin</li> <li>• Fetal platelet transfusion</li> </ul>                                                                                                                     |

splenic platelet pooling. Hematologic malignancies including the myeloproliferative disorders may result in marked splenomegaly. In particular, myelofibrosis may be associated with thrombocytopenia.<sup>6</sup> Portal hypertension from liver disease may also be associated with marked splenomegaly leading to thrombocytopenia or even pancytopenia. Cytokine-mediated mechanisms also contribute to the thrombocytopenia observed in liver disease.<sup>7</sup> The thrombocytopenia associated with splenic sequestration alone is generally moderate enough so as not to be associated with bleeding. However, concomitant conditions, such as the coagulopathy of liver disease or dysfibrinogenemia can exacerbate the tendency to bleed in this setting. Depending on the nature of the defect present, the use of Fresh Frozen Plasma to replace clotting factors and Cryoprecipitated AHF to provide fibrinogen may be important adjuncts to the use of platelet transfusion. Individuals with alcoholic cirrhosis and portal hypertension who continue

to consume alcohol may also have exacerbated thrombocytopenia resulting from suppression of platelet production from the megakaryocyte.<sup>8</sup>

Aside from splenomegaly, another potential cause of platelet sequestration is a cavernous hemangioma (Kasabach-Merritt syndrome). However, it is controversial whether platelet sequestration or platelet consumption is the primary defect leading to thrombocytopenia in this setting.<sup>9</sup>

### Congenital Platelet Disorders

Hereditary disorders may affect platelet number, platelet function, or both. Some congenital disorders leading to severe thrombocytopenia or markedly abnormal platelet function are identified early in life, but others are identified only after excessive bleeding is encountered in adulthood at the time of trauma or surgery.

### Congenital Megakaryocytic Hypoplasia

A markedly reduced platelet count early in life is seen with congenital megakaryocytic hypoplasia. This represents a group of disorders that include thrombocytopenia with absent radii syndrome.<sup>10</sup> These cases progress to include pancytopenia. Various mutations in the c-Mpl gene encoding the thrombopoietin receptor have been found.<sup>11</sup> Platelet transfusion is sometimes required in the event of bleeding because other therapeutic options such as corticosteroids, immune globulin, and splenectomy are ineffective. Ultimately, the definitive therapy to facilitate long-term survival appears to be hematopoietic stem cell transplantation.<sup>12</sup> An acquired form of megakaryocytic hypoplasia has been described that sometimes responds to immunosuppressive therapy.

### Platelet Receptor Defects

Glanzmann's thrombasthenia is an autosomal recessive condition associated with bleeding caused by absence, reduction, or impaired function of the glycoprotein (GP) IIb/IIIa receptor, which serves to bind fibrinogen and other cell adhesion molecules.<sup>13</sup> This receptor is also critical for sustained platelet activation in response to various agonists including epinephrine and adenosine diphosphate (ADP). The severity of bleeding in this disorder is quite variable, depending on the individual mutation present. Diagnosis is facilitated by platelet aggregation studies demonstrating a marked reduction or absence in platelet response to all standard agonists (ADP, epinephrine, collagen, arachadonic acid) except ristocetin.

Bernard-Soulier syndrome is an autosomal recessive condition associated with a variable reduction in platelet number and with the presence of large platelets.<sup>14</sup> It results from defects in the GPIb/IX/V complex that normally binds von Willebrand factor (vWF), resulting in platelet adhesion and activation. Similar to Glanzmann's thrombasthenia, the severity of bleeding is quite variable, depending on the exact mutation present. Diagnosis in this case is facilitated by the reduced platelet number, large platelets present on peripheral blood smear, and platelet aggregation assays demonstrating a response to all agonists except for ristocetin.

The platelet aggregation results in this disorder are thus complementary to the findings seen in Glanzmann's thrombasthenia.

### Other Congenital Defects Affecting Platelet Number or Function

Type 2B von Willebrand disease (vWD) is associated with an activating mutation in the von Willebrand protein that causes it to bind to platelets without the usual stimuli.<sup>15</sup> It is phenotypically indistinguishable from an activating mutation of the GPIb/IX/V receptor. This platelet-type vWD, or pseudo vWD, leads to binding of the platelet to vWF in the absence of activation of the usual signaling pathway. Both of these disorders result in reduction in platelet number because of clearance of activated or vWF-coated platelets in the spleen. Both are accompanied by a moderate bleeding tendency. Platelet aggregation assays performed using ristocetin demonstrate either spontaneous aggregation or heightened responsiveness to this agonist. Distinction between type 2B vWD and pseudo-vWD requires highly specialized laboratory testing.<sup>16</sup> Platelet transfusion must be administered very cautiously in patients with these disorders. In addition, use of desmopressin (DDAVP) is relatively contraindicated, as it may exacerbate thrombocytopenia. For bleeding in patients with type 2B vWD, use of a plasma-derived intermediate purity Factor VIII concentrate, such as Humate-P (CSL Behring, King of Prussia, PA) or Alphanate (Grifols, Barcelona, Spain) is recommended.

### Platelet Granule Defects

In addition to the surface receptors, platelets contain several types of granules that play important roles in aggregation, activation of coagulation, and wound healing. Hereditary defects in granule content, or abnormalities in the intracellular cyclooxygenase signaling mechanism may lead to impaired platelet function. These disorders include alpha granule deficiency (gray platelet syndrome is also associated with the Hermansky-Pudlak syndrome), dense granule deficiency, and combined deficiency.<sup>3,17</sup> Hereditary abnormalities in the cyclooxygenase signaling system are exceedingly rare. In these cases, platelet aggregation in response to many standard agonists is abnormal or abrogated, with the exception of aggregation to ristocetin, which is preserved.

### Therapy for Congenital Platelet Disorders

Although platelet transfusion is effective in the treatment of bleeding in congenital platelet disorders, it can lead to the production of platelet autoantibodies and thus should be reserved for serious bleeding episodes. Recombinant Factor VIIa (NovoSeven, Novo Nordisk, Bagsvaerd, Denmark) has been used effectively for Glanzmann's thrombasthenia in the setting of more severe bleeding, although it is not specifically approved for this indication.<sup>18</sup> Epsilon-aminocaproic acid (EACA) or DDAVP may be useful for minor bleeding or for perioperative management of minor procedures, depending on the severity of the underlying disorder. In the absence of any bleeding, patients should be counseled to avoid aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs),

and to take appropriate prophylactic measures before procedures (eg, use of EACA before minor dental procedures).<sup>19</sup>

### Acquired Platelet Disorders

Thrombocytopenia may be the primary manifestation of several different hematologic disorders. These include immune thrombocytopenic purpura (ITP) and the microangiopathic hemolytic anemias.

#### Immune Thrombocytopenic Purpura

Immune thrombocytopenic purpura (covered in more detail in Chapter 23) presents as an isolated reduction in the platelet count in the absence of abnormalities in the remaining cell lineages. In particular, red cell morphology is normal, and large platelets are often seen. In children, this condition is typically acute, is associated with an antecedent viral illness, and resolves spontaneously. In adults, chronic presentation is much more common.<sup>20</sup> Conditions associated with the development of ITP include human immunodeficiency virus infection, hepatitis C, and antiphospholipid antibodies.<sup>21</sup> The autoantibodies in ITP are most commonly directed against GPIIb/IIIa; however, they rarely affect platelet function.<sup>22</sup> In cases of severe or life-threatening bleeding, platelet transfusion is indicated. Although survival of transfused platelets is greatly reduced, they are likely to have some beneficial effect on active bleeding. Individuals with more moderate bleeding may be treated with immune globulin at doses of either 1 g/kg/day for 1 or 2 days or 0.4 g/kg/day for up to 5 days. In the absence of significant bleeding, corticosteroids represent first-line therapy in adult patients with ITP and platelet counts less than 30,000 to 50,000/ $\mu$ L. Corticosteroids may be administered either as 1 mg/kg of prednisone daily or dexamethasone 40 mg daily for 4 days.<sup>23</sup> Unfortunately, adult patients often relapse and require rescue therapies such as splenectomy or treatment with alternative immunosuppressive agents.

#### Microangiopathic Hemolytic Anemia

Several different conditions can cause a similar appearance on the blood smear: thrombocytopenia and the presence of red cell fragmentation (schistocytes).<sup>24</sup> However, clinical distinction between thrombotic thrombocytopenic purpura (TTP)/hemolytic uremic syndrome (HUS) and disseminated intravascular coagulation (DIC) is critical. In addition, malignant hypertension and valvular hemolysis may result in a similarly appearing smear.

TTP represents a medical emergency. Although the diagnostic pentad consists of fever, microangiopathic hemolytic anemia, thrombocytopenia, renal failure, and neurologic abnormalities, only microangiopathic hemolytic anemia and thrombocytopenia are required for the diagnosis.<sup>25</sup> Coagulation tests are almost always normal. The pathophysiology of this disorder has become elucidated as resulting from reduction or inhibition in function of the vWF-cleaving protease ADAMTS13.<sup>26</sup>

Platelets are activated in TTP, leading to a hypercoagulable state. Therefore, transfusion with platelets should be reserved for individuals with thrombocytopenia and life-threatening hemorrhage.

The therapy of choice is plasma exchange, although plasma infusion may be used as a temporizing measure. The use of antiplatelet agents and steroids is controversial. Relapsing TTP has been treated successfully with the CD20 monoclonal antibody rituximab.<sup>27</sup>

HUS in children is associated with diarrheal illness with shiga-toxin-producing bacteria such as *Escherichia coli* H:0157.<sup>28</sup> Supportive care is usually all that is required, because spontaneous recovery is the rule. In adults, however, HUS is additionally associated with use of various medications including cyclosporin A, gemcitabine, and mitomycin-C, among others. Use of potentially offending medications should be discontinued. Platelet transfusions are generally contraindicated in the absence of life-threatening bleeding as they are for TTP. However, the utility of plasmapheresis is less clear in this setting than for TTP, although successes have been reported, particularly in adults.

DIC is most commonly associated with infection or malignancy, although it can also be observed in the setting of vascular lesions such as hemangiomas and intravascular dissections. In contrast to TTP and HUS, the consumptive coagulopathy that occurs is not exacerbated by the administration of platelets and other blood components.<sup>29</sup> Although primary therapy should address the underlying condition, transfusions of platelets and other blood components may be safely administered in the interim.

### **Malignancy**

Thrombocytopenia is also a side effect of the cytotoxic chemotherapy administered for many different hematologic and nonhematologic malignancies. However, in addition, several hematologic malignancies are associated with inherent impairment in platelet function. Both the myelodysplastic and myeloproliferative syndromes are potentially associated with impaired platelet function out of proportion to any reduction in platelet number that is present. In this setting, platelet dysfunction results from ultrastructural abnormalities and impaired signaling, and may be manifest in platelet aggregation assays.<sup>30,31</sup> Platelet transfusions are sometimes indicated in the bleeding patient despite apparently acceptable platelet numbers. It should be noted, however, that myeloproliferative syndromes are also associated in some situations with platelet activation and thrombosis. Because of the latter, agents such as EACA should be used cautiously in these disorders and reserved for situations with reduced platelet function or severe thrombocytopenia.

### **Uremia**

Several nonmalignant acquired conditions are associated with platelet dysfunction. Of these, uremia is most commonly encountered in clinical practice. Elevated levels of metabolites, such as guanidinosuccinic acid, lead to impaired signaling from the surface receptors on the platelet through to cyclooxygenase.<sup>32</sup> Guanidinosuccinic acid also leads to inhibition of platelet aggregation by facilitating excess nitric oxide production.<sup>33</sup> Treatment of uremia with dialysis restores platelet function toward normal. For the patient with acute bleeding in the setting of uremia, several therapies have potential benefit.<sup>34</sup> DDAVP is administered

to release additional vWF from endothelial stores and to try to restore platelet signaling. It is reasonably safe and effective in this setting. Alternatively, administration of cryoprecipitate facilitates the same end. Recombinant Factor VIIa has been used off label in a small number of patients with severe bleeding in this setting, but its safety is still not well documented. For less acute bleeding, high doses of conjugated estrogens (several milligrams daily) may be effective. The mechanism of action is not well understood, and it usually takes several days to observe a beneficial effect.

### **Dialysis**

Although dialysis improves platelet function in uremia, it is also associated with the development of thrombocytopenia. In particular, continuous venovenous dialysis or hemofiltration has been associated with a reduction in platelet number in some patients.<sup>35</sup> It is important to distinguish thrombocytopenia caused by the dialysis procedure from heparin-induced thrombocytopenia, because patients are often treated with this anticoagulant in the intensive care setting.

### **Cardiac Interventions**

Cardiac procedures, use of ventricular assist devices, and cardiopulmonary bypass may all be associated with thrombocytopenia and/or defects in platelet function. Thrombocytopenia in cardiac catheterization is associated with the use of agents that inhibit GPIIb/IIIa function.<sup>36</sup> A hemorrhagic diathesis is exacerbated by the concomitant use of antiplatelet agents. Ventricular assist devices and cardiopulmonary bypass are also associated with platelet consumption.<sup>37</sup> As in the dialysis setting, the distinction of heparin-induced thrombocytopenia from other causes is critical. Platelet transfusion is indicated if simple platelet consumption is suspected, whereas it is relatively contraindicated when heparin-associated thrombocytopenia is present.

### **Medications and Supplements**

A number of medications, herbal remedies, and nutritional substances result in disorders of platelet function or number.<sup>38,39</sup> Aspirin and NSAIDs are among the most commonly used over-the-counter medications. In the case of aspirin, irreversible acetylation of cyclooxygenase in the platelet results in loss of function for the 5- to 7-day life span of the platelet. Conversely, NSAIDs are reversible inhibitors. This distinction is important in the bleeding patient. If a patient with life-threatening bleeding has consumed aspirin during the past several days, platelet transfusion may be indicated. However, in the case of NSAIDs, once at least four to five half-lives have elapsed, there is generally little utility in platelet transfusion unless a concomitant condition is present.

Immune-mediated thrombocytopenia (see Chapter 23) has been associated with the use of many different drugs. Most notably quinine, quinidine, sulfonamides, sulfonamide derivatives such as furosemide, and vancomycin have been associated with autoantibody formation.<sup>40,41</sup> Although platelet transfusion may

be necessary in patients with active bleeding, treatment consists primarily of discontinuation of the agent and observation. If necessary, treatment with immune globulin or corticosteroids may be initiated to hasten recovery of the platelet count.

Heparin-induced thrombocytopenia is a special class of immune-mediated thrombocytopenia in which antibodies form to the combination of heparin and platelet factor 4.<sup>42</sup> These antibodies bind to the platelet surface, resulting in activation and platelet clearance. The result of platelet activation is the presence of a highly hypercoagulable state that is not infrequently associated with either venous or arterial thrombosis. Although perhaps more commonly associated with unfractionated heparin, this syndrome is also associated with use of low-molecular-weight heparins. The diagnosis is suggested when the platelet count decreases by 50% or to less than 100,000/ $\mu$ L in a patient receiving one of these drugs. Although laboratory testing confirms the presence of antibodies, the diagnosis in the acute setting remains a clinical one. Because of the challenges inherent in distinguishing the cause of thrombocytopenia in critically ill patients, a relatively simple scoring system has been proposed.<sup>43</sup> When heparin-induced thrombocytopenia is suspected, treatment consists of immediate discontinuation of any heparin-containing product and institution of an alternative anticoagulant, such as lepirudin or the direct thrombin inhibitor argatroban. Use of warfarin alone is contraindicated, because it decreases protein C levels and can lead to catastrophic thrombosis.<sup>44</sup> Platelet transfusion is contraindicated even in patients with low counts on anticoagulation, except in the circumstance of life-threatening bleeding.

Many other commonly encountered classes of drugs are associated with qualitative or quantitative changes in platelets. These include traditional anticonvulsants such as valproic acid, which has also been associated with immune-mediated thrombocytopenia, and more recently approved agents such as gabapentin.<sup>45,46</sup> Simple discontinuation of these agents is generally all that is required in order to facilitate resolution of the defects. Drugs that affect both platelet number and function include heparin (mentioned above).

### Posttransfusion Purpura

The development of severe thrombocytopenia (platelet count often less than 10,000/ $\mu$ L) within approximately 1 week after blood transfusion should lead to suspicion of posttransfusion purpura (PTP). The pathophysiology of this disorder is poorly understood, but appears to involve autoantibody formation initiated by exposure to transfused antigens.<sup>47</sup> The most well-described cases involve autoantibodies to the platelet antigen HPA-1a ( $PI^{A1}$ ). Although the thrombocytopenia usually resolves spontaneously within a few weeks, there is a relatively high rate of bleeding associated with PTP, so that treatment should be considered. Platelet transfusion is generally ineffective. Administration of immune globulin (0.4 g/kg for 5 days or the equivalent) or plasmapheresis have been shown to have some efficacy in this setting, and represent reasonable therapeutic alternatives.<sup>48</sup>

### Neonatal Alloimmune Thrombocytopenia

About 1% to 2% of women have platelets of the HPA-1b ( $PI^{A2}$ ) phenotype. When they become pregnant with a fetus that has HPA-1a platelets, some of these women develop antibodies to HPA-1a. These antibodies cross the placenta and lead to fetal thrombocytopenia even during the first pregnancy, unlike the situation with Rh-negative individuals in which prior sensitization is required. Such fetal thrombocytopenia has been associated with intrauterine intracranial hemorrhage that usually occurs after about 30 weeks of gestation, as well as at the time of parturition. Most women come to attention after delivering a thrombocytopenic infant. Referral to specialists in high-risk obstetric practice is required. The administration of immune globulin after 20 weeks of gestation has sometimes been successful in preventing thrombocytopenia during further pregnancies.<sup>49</sup> In addition, use of intrauterine platelet transfusions may be considered (see Chapters 23 and 26).

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## Platelet Transfusion

### Platelet Products

In the United States, whole-blood-derived platelet units (also called random-donor units) are prepared as platelet-rich plasma (see Chapter 12).<sup>50,51</sup> First, red cells and platelet-containing plasma are separated. Then the platelet-containing plasma is spun at higher centrifugal force to generate platelet-poor plasma and a platelet pellet, which is resuspended in about 50 mL of plasma. Outside of the United States, the buffy coat method is often used for platelet preparation.<sup>52</sup> In this case, after centrifugation analogous to that used to separate plasma from the red cell pellet, the buffy coat layer atop the red cells is harvested. Both platelet preparation methods involve the pooling of four to six units, result in a similar quality product. Each unit contain at least  $5.5 \times 10^{10}$  platelets.<sup>53</sup> Thus, a 6-unit pool would contain  $3.3 \times 10^{11}$  platelets. This platelet dose generally leads to a platelet increment of 30,000 to 60,000/ $\mu$ L in the platelet-naïve patient when measured 1 hour after administration.

Apheresis platelets (often called single-donor platelets) are harvested from an individual through use of an apheresis device.<sup>54</sup> This procedure results in a platelet concentrate prepared from one donor that generally contains more than  $3 \times 10^{11}$  platelets. The potential advantage of this method of collection is that it reduces donor exposure. However, studies comparing apheresis platelets to leukocyte-reduced pooled whole-blood-derived platelets have not demonstrated any reduction in alloimmunization.<sup>55</sup> In addition, because of the highly sensitive nature of transmissible agent testing of units that is currently in place, any reduction in infectious risk is likely modest at best. In some areas of the United States, apheresis collection is significantly more expensive than whole-blood donation. Therefore, although the products may be considered interchangeable, use of whole-blood-derived platelets in some settings is preferred to use of apheresis platelets in the absence of special circumstances such as the need for HLA matching.<sup>56</sup>

## Platelet Storage and Processing

Platelets concentrates are stored at room temperature (22°C) because chilling activates them and results in a poor recovery after transfusion.<sup>57</sup> Storage at room temperature presents the challenge of bacterial contamination and growth. For these reasons concentrates are stored for up to 5 days. Storage for up to 7 days using current techniques and testing methodologies has generally shown similar product safety.<sup>58</sup> However, controversy over use of 7-day platelet storage still exists. Indeed, in early 2008 additional concerns over the possibility of bacterial contamination in 7-day stored platelets led to cessation of a Food and Drug Administration (FDA) Phase IV trial for production of 7-day stored platelets. As of this writing, the future of 7-day stored platelets is uncertain. Attempts to develop methods for refrigerating or freezing platelets have been made. However, to date none has been sufficiently successful to replace the current storage methods. The FDA has licensed a platelet system for prestorage pooling of whole-blood-derived platelets for storage up to 5 days. This system permits the same bacterial monitoring as is performed for apheresis platelets. See Chapters 12 and 49 for more details.

Following collection, platelet products can undergo leukocyte reduction (see Chapter 16). Although leukocyte reduction is associated with loss of up to 25% of the platelets in a concentrate, beneficial effects include reduction in cytomegalovirus (CMV) infection, febrile transfusion reactions, and platelet alloimmunization.<sup>59,60</sup> Gamma-irradiation that is used to prevent transfusion-associated graft-vs-host disease (GVHD) may decrease the platelet increment somewhat. However, it is certainly indicated in transplant recipients and other immunocompromised patients, including individuals with leukemia or lymphoma. (See Chapter 54 for more details.) Washing of platelet concentrates is time-consuming and may result in a decrease in platelet number.<sup>61</sup> Therefore, washing should be reserved for patients with severe allergic reactions who cannot be managed with appropriate medications.

## Platelet Dose

As noted above, a unit of whole-blood-derived platelets generally contains at least  $5$  to  $7 \times 10^{10}$  platelets and apheresis units contain more than  $3 \times 10^{11}$  platelets. In the absence of a consumptive coagulopathy, the platelet-naïve patient is expected to have a 1-hour platelet increment of 30,000 to 60,000/ $\mu$ L. Multitransfused patients have more modest increments. Both immune and nonimmune mechanisms are responsible for this phenomenon, although the latter appear predominant. In patients with acute myeloid leukemia who have received 25 platelet transfusions, the mean 1-hour platelet increment is reduced by about one-third and the 18- to 24-hour platelet increment is reduced by more than half in comparison to that observed after the first platelet transfusion.<sup>62</sup> Therefore, reduction in the number of unnecessary platelet transfusions is of significant benefit. However, the optimal platelet dose for each transfusion still remains to be determined.

Prophylactic transfusion refers to maintenance of the platelet count above a certain threshold in patients who are neither bleeding nor actively consuming platelets because of immune destruction or infection. Several recent studies have indicated that a patient platelet transfusion threshold of 10,000/ $\mu$ L is acceptable in the setting of stable patients with thrombocytopenia, including individuals with leukemia.<sup>63-65</sup> This is in relative agreement with older data estimating the fixed daily platelet production requirement for hemostasis to be about 7500/ $\mu$ L.<sup>66</sup> Potential benefits of the use of a threshold of 10,000/ $\mu$ L compared to higher platelet counts include a reduction in the incidence of platelet refractoriness and decrease in the incidence of transfusion-associated reactions.

Platelet transfusion is indicated in individuals with thrombocytopenia who are bleeding or who require invasive therapeutic interventions. The platelet transfusion threshold in such instances is dependent on the individual circumstances, including the presence of concomitant infection, coagulopathy, or a history of platelet refractoriness. Data from well-conducted clinical trials in this area are lacking, and in their absence only general guidelines can be provided.<sup>67</sup>

For mild bleeding, such as oozing from the gums or epistaxis, it is reasonable to maintain the platelet count above 20,000/ $\mu$ L. For more severe hemorrhage, such as gastrointestinal bleeding with a decrease in hemoglobin of at least 2 g/dL, maintenance of the platelet count above 30,000 to 50,000/ $\mu$ L is indicated. For intracranial hemorrhage, maintenance of the platelet count above 70,000 to 100,000/ $\mu$ L is recommended. Platelet counts of at least 50,000/ $\mu$ L are generally considered safe for general surgery. However, lower counts are accepted by some. Platelet counts of 70,000 to 100,000/ $\mu$ L are considered appropriate for neurosurgery.

## Platelet Administration

Platelets contain ABO antigens on their surface. Transfusion of ABO-mismatched platelets, however, is not associated with a major reaction. Nonetheless, there are data to support the use of ABO-matched platelet products whenever possible.<sup>68</sup> ABO matching appears to result in reduced immune destruction, and may have further implications as well. When the supply allows, ABO-matched platelets should be transfused.

In contrast to ABO, Rh blood group antigens are not expressed on the surface of the platelet. However, red cells with these antigens are invariably contained in platelet products. Therefore, transfusion of platelets from Rh-positive donors to Rh-negative recipients can lead to alloimmunization to the D antigen. Because of immune suppression, this is uncommonly encountered in patients with hematologic malignancies receiving Rh-mismatched platelets. The resolution to this issue is to administer matched products whenever available or to administer intravenous (IV) Rh Immune Globulin. A dose of 100 IU is generally protective for a transfused bag of platelets.<sup>69</sup>

Specific platelet transfusion indications, such as for patients with acute leukemia, lend themselves to the use of leukocyte-reduced, irradiated platelet products (see Chapters 16 and 54).

Because of the potential reduction in the number of platelets, washing of platelets should be reserved for individuals with severe allergic reactions that cannot otherwise be overcome by appropriate medications.

Although acetaminophen 650 to 1000 mg and diphenhydramine 25 to 50 mg are frequently administered before platelet transfusion as primary prophylaxis against febrile and allergic reactions, few data exist to support this as routine practice.<sup>70,71</sup> However, once an allergic reaction has been observed (hives, wheezing, dyspnea), diphenhydramine is the drug of choice to prevent additional reactions in the future. If a moderate allergic reaction recurs in an adult despite administration of these premedications, hydrocortisone 100 mg IV, is often effective. Additional agents that might be of benefit include H<sub>2</sub>-antagonists such as cimetidine or ranitidine and doxepin. Recurrence of severe allergic reactions despite premedication is an indication for the use of washed platelets.

Platelet concentrates must be administered to adults through a blood filter. Generally a standard blood set with a 170- or 260-micron filter, or a platelet leukocyte reduction filter is used, with an infusion time of about 30 minutes to 1 hour. Slower administration, such as a continuous platelet drip, may be desirable in some settings. However, once the bag is entered the contents must be administered within 4 hours. Use of a mechanical pump has the advantage of a more controlled rate of platelet infusion and should be considered if a continuous platelet drip is employed.<sup>72</sup>

Platelet transfusion is not without risk. Aside from the development of platelet refractoriness and allergic reactions that can be observed with any blood component, platelets are the component most commonly associated with the transmission of bacterial infection.<sup>73</sup> In the past it has been estimated that about 1 in 5000 platelet units are contaminated with bacteria. Newer standards for bacterial testing should help reduce the incidence of bacteremia associated with platelet transfusion. Nonetheless, there is a heightened sense of awareness for the possibility of bacterial transmission in those receiving platelet transfusions, particularly if individuals are neutropenic and become febrile during or shortly after a platelet transfusion.

## Platelet Refractoriness

### Determination

Platelet refractoriness is often defined as failure to achieve the expected increment in the platelet count after two consecutive transfusions.<sup>74</sup> A poor response to a given platelet unit is common.

Determination of platelet refractoriness requires a platelet count between 10 and 60 minutes following completion of the transfusion. Lack of a response at a later time following transfusion can be caused by underlying clinical problems, whereas the absence of a response at this immediate point after transfusion usually indicates refractoriness from immunologic causes.

Two methods that are often used in the setting of clinical research are calculation of the corrected count increment

(CCI) and the 1-hour posttransfusion percent platelet recovery (PPR).<sup>75</sup> The formulas for these are as follows:

$$CCI = \frac{(\text{post-tx plt count}/\mu\text{L} - \text{pre-tx plt count}/\mu\text{L}) \times \text{BSA in m}^2}{(\text{number of plts transfused}) \times 10^{-11}}$$

$$PPR = \frac{(\text{post-tx plt count}/\mu\text{L} - \text{pre-tx plt count}/\mu\text{L}) \times \text{weight in kg} \times 75 \text{ mL}}{\text{plt count of product transfused}/\mu\text{L} \times \text{volume of plts in mL}}$$

where BSA is the body surface area, plt is platelet, and tx is transfusion. Although the number of platelets or platelet count of the product transfused is generally not known, approximations may be used. As noted earlier, a unit of whole-blood-derived platelets generally contains at least  $5.5 \times 10^{10}$  platelets. Thus a 6 unit pool contain  $3.3 \times 10^{11}$  platelets and units collected by apheresis contain at least  $3 \times 10^{11}$  platelets. These values can be used in the calculation. In a 75-kg individual a CCI of 10,000 to 20,000/ $\mu\text{L}$  is expected. Lower values than these are consistent with refractoriness.

An alternative calculation used in daily clinical practice is the platelet increment (posttransfusion platelet count/ $\mu\text{L}$  – pretransfusion platelet count/ $\mu\text{L}$ ). Assuming a platelet recovery of 60% in this setting, a whole-blood-derived platelet pool or an apheresis unit administered to a thrombocytopenic patient should increase the platelet count on average by about 20,000 to 40,000/ $\mu\text{L}$ .<sup>76</sup>

### Causes

Lack of response to platelet transfusion may be the result of an underlying acute illness, such as infection, or may result from a more chronic condition, such as splenomegaly. Fever, sepsis, DIC, hemorrhage, and conditions associated with marrow transplantation such as veno-occlusive disease and GVHD all contribute to platelet refractoriness.<sup>77</sup> Treatment of the underlying illness is indicated. Independent of other nonimmune and immune causes of decreased recovery, for reasons that are poorly understood, repeated platelet transfusion may be associated with decreased platelet recovery in certain populations, such as individuals with acute myeloid leukemia. Nonimmune causes generally have their greatest impact on the response to platelet transfusion beyond the 60 minutes and reduce, but do not eliminate, responsiveness.

Because platelets contain a variety of surface antigens, including ABO antigens, HLA antigens, and platelet-specific antigens, exposure to allogeneic transfusions can result in the formation of antibodies. Platelet refractoriness is, therefore, more commonly observed in patients who have received multiple transfusions and/or been pregnant.<sup>78</sup>

The most commonly encountered antibodies are those to HLA antigens (about 45%) and not to other platelet-specific antigens. However, compelling randomized data from patients undergoing treatment for acute leukemia or autologous transplantation for Hodgkin lymphoma have suggested that transfusion of

ABO-incompatible platelets increases the incidence of platelet alloimmunization. ABO incompatibility is defined in this case as the presence of A or B antibodies in the recipient to donor A or B antigens. Nine of 13 patients (69%) receiving ABO-incompatible platelets became platelet refractory with a median onset at Day 15 compared with only one of 13 patients (8%) treated with compatible platelets ( $p < 0.001$ ).<sup>79</sup> Mechanisms proposed include development of antibodies to the mismatched antigen and nonspecific stimulation of the immune system. Additional retrospective data in the literature support this finding. Use of ABO-matched platelets is recommended when possible, particularly for patients with hematologic malignancies who are likely to receive numerous platelet transfusions over time.

## Management of Platelet Refractoriness

### Platelet Crossmatching and HLA Matching

Platelet crossmatching and HLA matching may be used effectively to overcome platelet refractoriness (Fig 13-1). Each strategy has its advantages and disadvantages (see Chapters 11 and 31 for more detail).<sup>80</sup> Platelet crossmatching allows for screening of the transfused platelet product against HLA antigens and platelet glycoprotein antibodies. Because it does not rely on HLA typing of the recipient or specific donor procurement, it is potentially more rapid and less expensive than HLA typing. However, the number of units available for testing is often limited.

HLA matching of platelets uses databases of HLA-typed platelet donors who can then be asked to donate by apheresis. This requires logistic coordination, with a time lag of approximately 48 hours to facilitate appropriate transmissible agent testing, and is not effective in the 5% to 10% of patients who have antibodies to other platelet glycoproteins.

Despite use of crossmatched or HLA-matched platelet units, certain individuals remain platelet refractory. In some of these cases, resolution of concomitant conditions such as sepsis or hemorrhage results in a return of platelet responsiveness. In others, the underlying mechanism involves nonimmune mechanisms facilitating rapid platelet clearance.

### Continuous Infusion

For the patient who is actively bleeding or at high risk of bleeding because of severe refractory thrombocytopenia (platelet count less than 5,000 to 10,000/ $\mu\text{L}$ ), the use of a continuous infusion of a low dose of platelets may be employed. The equivalent of about three whole-blood-derived platelet units is administered over 4 hours, ideally with use of a mechanical pump. Even in the absence of a measurable increase in the platelet count this approach may be effective in facilitating hemostasis. Another advantage to this approach over repetitive bolus administration used in an attempt to achieve a measurable platelet increment is that it provides the blood bank with reasonable inventory

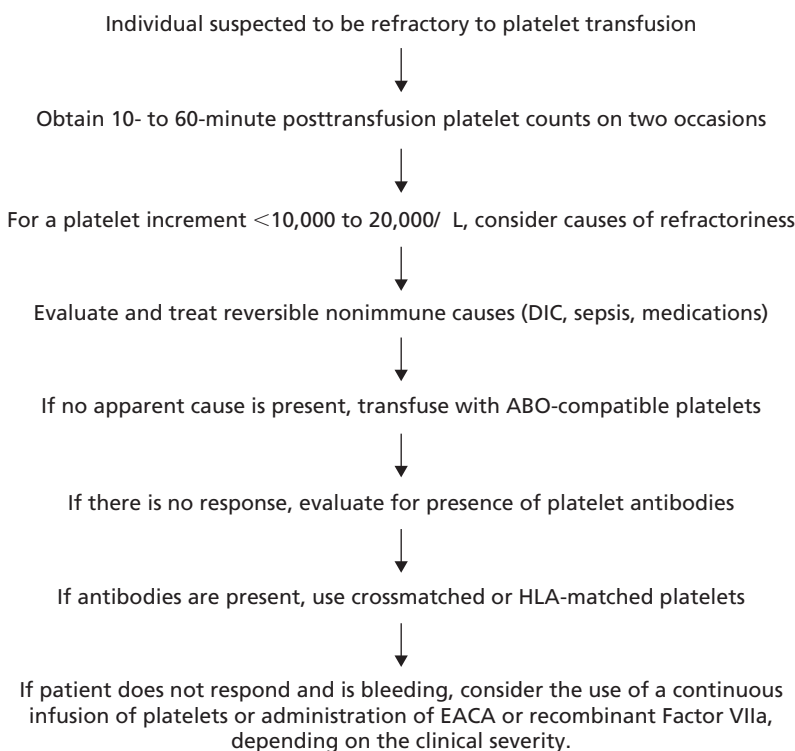


**Figure 13-1.** Evaluation and management algorithm used for individuals who appear to be refractory to platelet transfusion.  
DIC = disseminated intravascular coagulation;  
EACA = epsilon-aminocaproic acid.

expectations. Because of the significant blood bank and nursing resources involved, the use of a continuous infusion of platelets should be reserved for appropriate refractory cases in which alternatives have been thoroughly evaluated.

### Other Modalities

The administration of immune globulin has been reported in certain cases to be successful in overcoming platelet refractoriness.<sup>81</sup> Doses used have often been similar to those used in ITP (0.4 g/kg daily for 5 days or its equivalent). However, there are no data more systematically examining this modality.

EACA and tranexamic acid are effective adjuncts to platelet transfusion in patients with severe thrombocytopenia and bleeding (tranexamic acid is not available in the United States). Even at relatively low doses of 1 g every 6 hours, EACA appears to be effective in this setting. In one retrospective study of thrombocytopenic patients who were bleeding, 51 of 77 patients (66%) achieved a complete response to EACA administration, resulting in a reduction in platelet and red cell transfusion requirements.<sup>82</sup> Although antifibrinolytic agents should not be used in patients with DIC or upper urinary tract bleeding, their safety and efficacy profile in other thrombocytopenic patients with hemorrhage is favorable.

Sometimes touted as a universal hemostatic agent, recombinant Factor VIIa has been used in the setting of severe thrombocytopenia with bleeding in order to achieve hemostasis.<sup>83</sup> At this time, it is reasonable to consider the use of recombinant Factor VIIa at a dose of approximately 90 µg/kg for the thrombocytopenic individual who is refractory to platelet transfusion and has life-threatening bleeding. Aside from this setting, the most systematic data for use of this agent in platelet disorders exists for those with Glanzmann's thrombasthenia. It has been suggested that 90 µg/kg given to these individuals every 2 hours for three or more doses is effective in controlling bleeding.<sup>84</sup> Although observed to be effective in some cases, clinical trials are required to better define the use of this agent in those with thrombocytopenia or platelet function disorders.

### Alternatives to Platelet Transfusion

Current storage constraints on platelet concentrates often result in shortages of this product. Attempts have been made to develop platelet substitutes and novel storage technologies.<sup>85</sup> Although some promising approaches have been used, such products are not yet available for routine clinical use. Conversely, one cytokine [interleukin 11 (IL-11)] has received regulatory approval for use in the management of thrombocytopenia and several other agents are likely to be approved in the near future.

### Platelet Substitutes and Storage Technologies

Platelet substitutes are in relatively early stages of development. Among other approaches, ongoing efforts are being made to develop human platelet fragments that could be stored for lengthy periods and platelet cell surface molecules that are coupled to nanoparticles.<sup>86</sup>

The lesion that leads to the clearance of platelets that have been stored in the cold (below 12°C) has been investigated extensively. In vitro, it appears that glycosylation (in particular, galactosylation) can prevent the clearance of refrigerated human platelets that are otherwise recognized by macrophage complement type 3 (CR3) receptors.<sup>87,88</sup> Whether modification of platelets in this manner will facilitate the effective transfusion of platelets in humans remains to be seen.

### Cytokines and Thrombopoietin Analogs

Recombinant IL-11 is a growth factor produced by DNA technology that stimulates proliferation of hematopoietic stem cells (including megakaryocytes), induces megakaryocyte maturation, and increases platelet production. IL-11 was found to be moderately effective in reducing the need for platelet transfusions when administered subcutaneously at a dose of 50 µg/kg daily in patients receiving cancer chemotherapy that caused severe thrombocytopenia.<sup>89</sup> However, these trials were relatively small. IL-11 has been associated in some individuals with allergic reactions, and it has also been associated with cardiovascular adverse events including atrial arrhythmias and tachycardia.

Because of the promise of more specific and potent stimuli for platelet production, the investigation of thrombopoietin analogs has overshadowed the development of IL-11. Thrombopoietin was identified as the 332 amino acid glycoprotein ligand for the c-Mpl receptor.<sup>90</sup> Although it has effects on other stem cells, it has been shown to stimulate platelet production through the proliferation of marrow stem cells and the maturation of megakaryocytes. Thrombopoietin is regulated through a feedback loop. It is synthesized primarily in the liver and binds to the surface of circulating platelets and to megakaryocytes. When thrombocytopenia is present, more thrombopoietin is available to bind to the c-Mpl receptor and stimulate platelet production. Initial attempts at the development of a thrombopoietin analog for therapeutic use (pegylated recombinant megakaryocyte growth and development factor) were associated with antibody formation in healthy volunteer platelet donors. Thus, further development was halted. However, more recently a recombinant thrombopoietin analog has been developed in which part of the molecule has been fused to an immunoglobulin Fc peptide. This molecule, AMG531, has been termed a peptibody. Studies conducted in patients with chronic ITP demonstrated that when administered subcutaneously, this agent was safe and effective, producing platelet responses in the range of 50,000 to 450,000/µL in more than half of the patients treated with an acceptable safety profile.<sup>91,92</sup> The FDA granted regulatory approval of this agent in 2008.

Other approaches to stimulating platelet production have involved developing nonpeptide molecules that stimulate the c-Mpl receptor. Several of these compounds are currently in development. The most advanced of these is eltrombopag. This oral agent has been evaluated in patients with ITP and in patients with thrombocytopenia resulting from cirrhosis and hepatitis C virus infection. In both settings it was highly effective in a



dose-dependent manner when compared with placebo in increasing the platelet count to the target range. At a dose of 75 mg daily, 81% of patients with ITP achieved a platelet count of greater than 50,000/ $\mu$ L and 65% of patients with cirrhosis and hepatitis C infection achieved a platelet count of greater than 100,000/ $\mu$ L.<sup>93,94</sup> Additional studies with AMG531, eltrombopag, and other novel investigational agents are ongoing.

Although efficacy in settings such as hematopoietic stem cell transplant is unlikely given the above-noted experience, other applications are possible. For example, AMG531 has been reported to result in improvement in platelet counts in patients with myelodysplastic syndromes.<sup>95</sup> However, given the emerging safety data regarding another hematopoietic growth factor, erythropoietin, use of thrombopoietin-mimetics in the setting of malignancy will likely proceed in a cautious manner.<sup>96</sup>

## Summary

A variety of diverse congenital and acquired conditions are associated with thrombocytopenia or its functional equivalent. Congenital causes are rare, but acquired causes are relatively common. In particular, thrombocytopenia or platelet dysfunction associated with the administration of chemotherapy or with surgical procedures are leading indications for platelet transfusion. The availability of a variety of platelet products for transfusion facilitates the supportive care of individuals receiving chemotherapy and the treatment of bleeding individuals with thrombocytopenia or its functional equivalent. Leukocyte-reduced whole-blood-derived platelets and apheresis platelets are associated with similar rates of infection and alloimmunization, and can be used interchangeably in most settings. A prophylactic platelet transfusion threshold of 10,000/ $\mu$ L is generally acceptable for afebrile patients receiving cancer chemotherapy. Maintenance of a higher platelet count is indicated in a number of situations, including the settings of sepsis, DIC, hemorrhage, trauma, and surgery. Febrile and allergic reactions to platelet transfusion can often be managed conservatively with the administration of acetaminophen and diphenhydramine. Platelet refractoriness based on nonimmune and immune mechanisms remains a major issue when repeated transfusions are required. After platelet refractoriness following two transfusions has been documented, ABO matching, platelet crossmatching, and HLA matching may be used as initial strategies to address the situation. Antifibrinolytic agents and recombinant Factor VIIa are useful adjuncts to platelet transfusion in the bleeding or platelet refractory patient. Because of the limitations of current storage techniques, research and development continue on platelet storage and substitutes. Thrombopoiesis-stimulating agents should provide additional therapeutic alternatives for the management of thrombocytopenia in specific settings. However, much additional research and development will be necessary in order to establish their safety and efficacy in patients receiving cancer chemotherapy.

## Disclaimer

The author has disclosed no conflicts of interest.

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## 14

# Neutrophil Production and Kinetics: Neutropenia and Neutrophilia

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The neutrophil is the body's principal defense against bacterial infection and is especially equipped to locate, phagocytose, and kill invading microorganisms. The system of neutrophil production in the marrow and distribution in the blood and tissues serves both to maintain a baseline supply of these cells and to ensure the rapid delivery of increased numbers of neutrophils to areas of infection or inflammation.

## Production in Marrow

The pluripotent stem cell may give rise to any type of hematopoietic cell. With differentiation, a fraction of these cells becomes committed to the neutrophil line. Development beyond this committed progenitor stage proceeds sequentially from the myeloblast, the earliest recognizable neutrophil precursor, to the promyelocyte, the myelocyte, the metamyelocyte, the band forms, and the mature segmented neutrophil, at which time cells are released into the circulation—a process that normally takes 10 to 12 days. There are two important features to marrow neutrophil production: cell proliferation and cell maturation. Cell division is limited to the myeloblast, promyelocyte, and myelocyte compartments.<sup>1</sup> Most cells probably undergo four or five divisions between the myeloblast and myelocyte stage, each division taking approximately 1 day.<sup>2</sup> Cell maturation occurs continuously from the myeloblast to the mature neutrophil. An essential feature of this process is the ordered development of the neutrophil's characteristic four types of cytoplasmic granules. Primary granules containing myeloperoxidase and serine proteases appear during the promyelocyte stage; secondary granules containing lactoferrin and collagenase and tertiary granules containing gelatinase appear at the myelocyte stage; and secretory vesicles appear at the stage of the mature cell. This cytokine-driven chronology is based on the transcriptional regulation of sequentially expressed genes coding

the various granular proteins.<sup>3</sup> Additional changes during maturation include increased adherence abilities, increased deformability, increased responsiveness to chemoattractants, and the development of the neutrophil's characteristic segmented nucleus. Under normal circumstances the transit time for the postmitotic pool is approximately 7 days,<sup>4</sup> a value that can be shortened by 2 to 3 days under situations of stress.<sup>5</sup> Normal baseline neutrophil production, based on the transit time and the size of the postmitotic compartment, is approximately  $0.85 \times 10^9$  cells/kg/day.<sup>4</sup>

An important requirement of the neutrophil defense system is that it be able to deliver large numbers of mature neutrophils to a site of infection or inflammation. To this end, approximately two-thirds of the marrow postmitotic pool, composed of segmented neutrophils and band forms, can be shifted rapidly from the marrow into the circulation under situations of stress or of increased tissue neutrophil demand.<sup>6</sup> Mobilization also occurs as a response to stimuli such as exercise, corticosteroids, endotoxin, cytokines such as granulocyte colony-stimulating factor (G-CSF), chemokines, or bacterial products.<sup>7,8</sup> The time course varies with the stimulus from a few minutes (chemokines) to a few hours (G-CSF, corticosteroids, infection). For G-CSF-mediated mobilization, the interaction of the chemokine stromal-derived factor-1 (SDF-1) and its receptor (CXCR4) appear to be key.<sup>8</sup> SDF-1 is constitutively produced by osteoblasts and marrow stromal cells. CXCR4 is expressed on neutrophils, and retention in the marrow may depend on this SDF-1/CXCR4 interaction. With G-CSF administration, marrow SDF-1 levels and neutrophil CXCR4 levels both fall dramatically. Treatment with AMD3100, an inhibitor of CXCR4, results in neutrophil mobilization. Regardless of the mechanism, the extent of the storage pool shift depends on the intensity of the stimulus and will be reflected in the magnitude of the neutrophilia and in the degree to which immature forms are seen in the blood.

## Regulation of Neutrophil Production

The commitment of a pluripotent progenitor to myeloid development is incompletely understood but is strongly influenced by

both cytokines and transcription factors. The cytokines G-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3, and IL-6 all stimulate granulopoiesis, but G-CSF appears to be pivotal.<sup>8</sup> In addition to inducing commitment to the myeloid line, G-CSF stimulates myeloid proliferation, reduces transit time through the marrow, and causes release of neutrophils from the marrow. Knockout mice for either G-CSF or the G-CSF receptor exhibit severe neutropenia,<sup>9,10</sup> as do animals treated with antibody to G-CSF.<sup>11</sup> In vivo, G-CSF causes a dramatic increase in neutrophil production with marked neutrophilia.<sup>5,12</sup> Cell development along the neutrophil line is also dependent on more than a dozen transcription factors, molecules that bind to DNA and regulate gene expression.<sup>13</sup> Two of the critical factors are PU.1 and CCAAT/enhancer-binding protein  $\alpha$  (C/EBP $\alpha$ ). PU.1 binds to promoter sequences and is essential for maturation of cells in the myeloid lines. C/EBP $\alpha$  is important in early myeloid development. C/EBP $\alpha$  null mice lack mature neutrophils, and the cells do not express the G-CSF receptor. Presumably there is a negative feedback system in which inflammatory stimuli or a paucity of mature neutrophils stimulates neutrophil production. Stark et al<sup>14</sup> have recently described such a system in mice in which tissue phagocytosis of apoptotic neutrophils reduced IL-23 production by macrophages/dendritic cells. IL-23 normally stimulates T cells to produce IL-17 which, in turn, stimulates production of G-CSF. Thus, adequate numbers of tissue neutrophils would decrease G-CSF levels, whereas a relative lack of tissue neutrophils would increase G-CSF levels, and stimulate neutrophil production.

## Blood Kinetics

Neutrophils leave the circulation by first-order kinetics, implying random loss rather than loss caused by senescence, a finding consistent with the fact that these cells perform their primary function after they leave the bloodstream. The blood half-disappearance time is normally 6 to 8 hours.<sup>4,15</sup> Blood neutrophils are distributed between two freely exchangeable pools of approximately equal size, the circulating pool and the marginal pool.<sup>16</sup> Cells in the circulating pool are those readily sampled by drawing blood. Conventionally, the cells in the marginal pool have been considered to be loosely adherent to the endothelium of the small venules. This notion has arisen from direct observation of the microcirculation in animals, which has shown neutrophils slowly rolling along the endothelial surfaces outside the main axial stream of the blood flow<sup>17,18</sup> as well as from the observation that cells in the marginal and circulating pools are in rapid equilibrium.<sup>16</sup> However, other information suggests that the marginal pool is not diffusely distributed throughout the vasculature but is largely localized to the liver, the spleen, or the pulmonary vasculature.<sup>19-25</sup>

## Migration into Tissues

The process of migrating from the blood to a site of inflammation is complex.<sup>24-26</sup> The initial stage of neutrophil capture and

rolling along the endothelial surface is mediated by the interaction of selectins and their ligands. L-selectin on the neutrophil surface interacts with a sialomucin oligosaccharide on the endothelial cell surface.<sup>24-27</sup> The ligand for P-selectin and E-selectin on the endothelial cells is platelet sialoglycoprotein ligand-1 (PSGL-1). The slow rolling of the neutrophils, in concert with local inflammatory signals, initiates firm adhesion, mediated by the interaction of neutrophil  $\beta 2$  integrin CD11/18 with intercellular adhesion molecule 1 (ICAM-1) on the endothelial cells.<sup>24-27</sup> Neutrophils migrate between endothelial cells into the tissues; they home to these migration points via an IgG-type molecule, platelet-endothelial cell adhesion molecule 1 (PECAM-1). The importance of these adhesion molecules for neutrophil emigration from the blood has been demonstrated by the effects of antibodies to these proteins<sup>25,28,29</sup> and by the identification of patients who lack these molecules or their ligands.<sup>30-33</sup> Patients with leukocyte adhesion deficiency types I and II are deficient in the  $\beta 2$  integrin and in a selectin ligand (SLeX), respectively, on the neutrophil surface. Both disorders are characterized by severe neutrophil dysfunction, neutrophilia, an inability to deliver neutrophils to sites of infection, and recurrent bacterial infection.

The nature and mechanisms of neutrophil margination and emigration appear to be different in the pulmonary vasculature than in the systemic vasculature. Neutrophil sequestration and migration in the lungs occurs principally in the capillary bed rather than in the post-capillary venules.<sup>25</sup> The degree of neutrophil sequestration in the pulmonary capillary bed, 35 to 100 times that of the larger systemic vessels, may be more related to cell size and deformability than to the influence of adhesion proteins.<sup>34-36</sup> The role of the adhesion proteins in neutrophil migration to the alveolar space may depend on the particular inflammatory stimulus. Recent evidence has indicated that emigration after infection with gram-positive organisms is independent of selectin/integrin, whereas that seen after infection with gram-negative organisms requires the involvement of these adhesion proteins.<sup>25,35,37</sup>

Neutrophil survival in both the blood and the tissues depends upon the rate of cellular apoptosis, the regulation of which is poorly understood. Pro-inflammatory stimuli such as G-CSF inhibit apoptosis, prolonging cell survival both in vitro and in vivo.<sup>5,38</sup> Pro-apoptotic factors, which serve to dampen the inflammatory response, have been identified in certain tissue inflammatory sites. Regulation of neutrophil apoptosis thus appears to be an important determinant of the intensity of the inflammatory process.<sup>39</sup>

## Response to Infection

The kinetic features of the inflammatory response are shown in Fig 14-1. The first phase is characterized by a rapid shift of bands and segmented neutrophils from the marrow into the blood, typically associated with an increase in the degree of margination. At the same time, expansion of cell production in the mitotic pool occurs by an increased rate of division of promyelocytes and myelocytes. It then takes 2 to 4 days for the leading edge of this wave of proliferation to proceed through the maturational pool

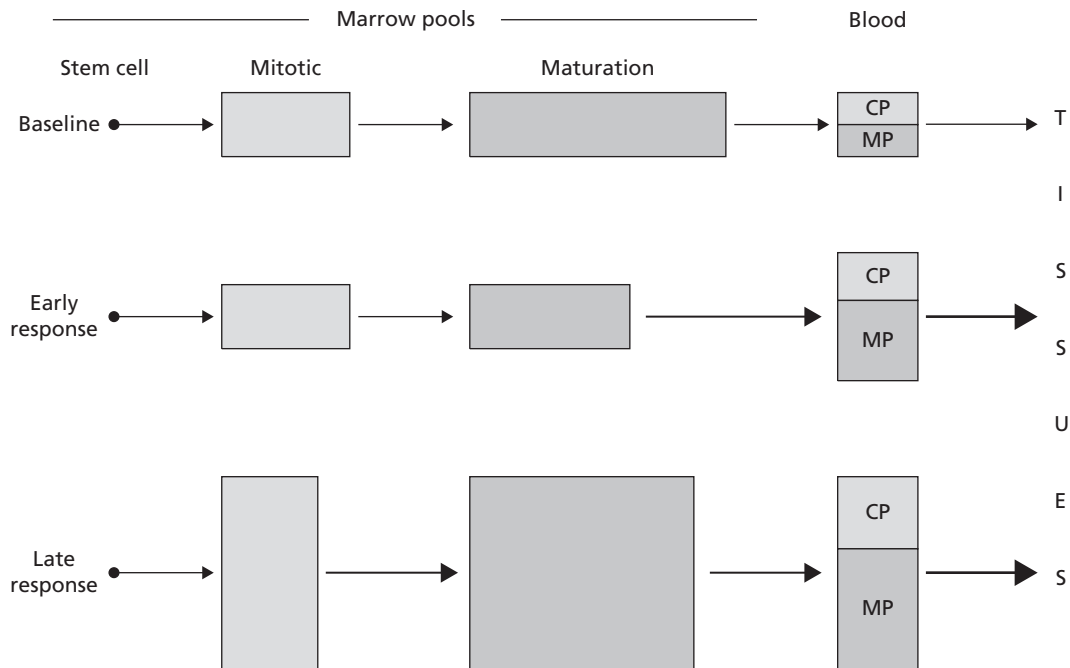


**Figure 14-1.** Marrow response to inflammation and/or infection. Pool size is represented by height of the box. Time spent in each pool is represented by width of the box. Rate of cell flow between compartments is represented by size of the arrows. Mitotic pool consists of myeloblasts, promyelocytes, and myelocytes. Maturation pool consists of metamyelocytes, bands, and segmented neutrophils. Early response is characterized by shift of marrow storage pool into circulation and increased margination of blood neutrophils. Late response is characterized by proliferation of mitotic pool and reduced transit time through the marrow pools. CP = circulatory pool; MP = marginal pool.

and reach the blood, resulting in a steady increased supply of neutrophils for delivery to the tissues. The degree to which marrow neutrophil production can increase during a serious infection has never been determined. However, if one assumes that, as with other cell lines, production might be able to increase five-fold, one might expect peak to neutrophil marrow output to be as much as  $5 \times 10^9$  cells/kg/day. When the infection clears or the inflammatory process runs its course, the marrow proliferative activity returns to baseline over the period of a few days.

## Blood Neutrophil Count

Although blood is merely the vehicle for transporting neutrophils from the marrow to the tissues, the blood neutrophil count remains the physician's most convenient measure of the status of the neutrophil defense system. Abnormalities of the neutrophil count are caused by alterations in the rate of effective marrow production, shifts from the marrow reserve into the circulation, or changes in the distribution or survival of blood neutrophils.

## Neutropenia

### Classification

Neutropenia is defined as a blood neutrophil count less than  $1500/\mu\text{L}$ . Neutropenia is important not only because it is an

indicator of an underlying abnormality but also because it can be associated, if severe (less than  $500/\mu\text{L}$ ), with an increased incidence of bacterial or fungal infection. It occurs in a variety of infectious, inflammatory, nutritional, malignant, and hematologic diseases, and as a consequence of many chemotherapeutic agents. In most of these disorders neutropenia is accompanied by significant degrees of anemia and/or thrombocytopenia. The following discussion focuses on the selective neutropenias, those conditions in which neutropenia is the dominant hematologic abnormality (Table 14-1).

The most common cause of isolated neutropenia is drug related. Cytotoxic drugs used for treatment of malignancy or for immunosuppression regularly cause neutropenia and, if given in large enough doses, will do so in all patients. These treatment-induced neutropenic states are further discussed in Chapters 15 and 31. Neutropenia can also result from an idiosyncratic reaction to a drug that, in most patients, does not cause neutropenia. In severe cases, the onset is sudden, and patients present with high fever, malaise, and perhaps evidence of localized infection. Blood neutrophils are few or absent; marrow examination reveals depletion of mature neutrophils and, in severe cases, absence of all myeloid cells. Many drugs have been reported to be responsible for these reactions.<sup>40-42</sup> These drug reactions are thought to occur by one of two mechanisms. The first is dose-related inhibition of neutrophil development, either by direct suppression of precursors (eg, ticlopidine, chlorpromazine) or by induction of apoptosis (eg, clozapine). The second is an immunologic

Table 14-1. Selective Neutropenias

| Cause                                 | Syndrome                                                                                                                                                                                                                                                                       |
|---------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Drug Induced</b>                   | Cytotoxic<br>Idiosyncratic                                                                                                                                                                                                                                                     |
| <b>Decreased Marrow Production</b>    |                                                                                                                                                                                                                                                                                |
| • Congenital                          | Severe congenital neutropenia<br>Myelokathexis<br>Shwachman-Diamond syndrome<br>Chediak-Higashi syndrome<br>p14 deficiency<br>Cyclic neutropenia<br>Type 1b glycogen storage disease<br>Reticular dysgenesis<br>Dyskeratosis congenita<br>Barth syndrome<br>Griscelli syndrome |
| • Chronic idiopathic neutropenia      | Various congenital/acquired disorders                                                                                                                                                                                                                                          |
| • Acquired                            | Nutritional deficiencies<br>Pure white cell aplasia                                                                                                                                                                                                                            |
| <b>Increased Utilization/Turnover</b> |                                                                                                                                                                                                                                                                                |
| • Alloimmune                          | Isoimmune neonatal neutropenia                                                                                                                                                                                                                                                 |
| • Autoimmune                          | Idiopathic<br>Systemic lupus erythematosus<br>Rheumatoid arthritis<br>Sjögren syndrome                                                                                                                                                                                         |
| <b>Increased Margination</b>          | Infection/inflammation<br>Splenomegaly                                                                                                                                                                                                                                         |

reaction (eg, aminopyrine, propylthiouracil), the patient having developed an antibody to the drug or a drug metabolite. The two mechanisms are not distinguishable on the basis of the original clinical presentation. In either case, the offending drug should be considered permanently contraindicated.

The congenital neutropenias comprise a diverse collection of syndromes characterized by decreased marrow neutrophil production.<sup>43-46</sup> The pathophysiologic mechanisms are incompletely understood, but a common feature appears to be increased apoptosis of marrow neutrophil precursors. The best-studied syndrome has been that of severe congenital neutropenia. This disorder presents in infancy and is characterized by severe neutropenia (<500 cells/ $\mu$ L) and repeated episodes of infection. Examination of the marrow reveals few cells beyond the promyelocyte stage. Before the availability of G-CSF treatment, most patients did not survive childhood. Plasma G-CSF levels are normal or elevated, and G-CSF receptor expression is normal. Inheritance may follow an autosomal dominant or recessive pattern, the latter comprising about one-third of the cases. Mutations in the gene for neutrophil elastase (ELA2) are present in about two-thirds of the autosomal dominant or sporadic cases, but elastase RNA and protein levels are decreased in all patients, whether or not an ELA2 mutation is present. Mutations in the *HAX1* gene have been detected in the autosomal recessive

variety.<sup>47</sup> To date, all patients studied have exhibited downregulation of the downstream transcription factor lymphoid enhancer-binding factor 1 (LEF-1). LEF-1 interacts with both C/EBP $\alpha$  and the ELA2 promoter and is required for the maturation of myeloid precursors.

Myelokathexis is a disorder in which severe neutropenia is accompanied by a hypercellular marrow containing neutrophils with hypersegmentation and degenerative changes. The underlying pathology appears to be accelerated neutrophil apoptosis with resultant ineffective marrow neutrophil production. The genetic basis for this disorder is a mutation of the gene coding for the chemokine receptor CXCR4.<sup>45</sup> The disorder may be part of the WHIM syndrome, characterized by warts, hypogammaglobulinemia, infection, and myelokathexis.

Shwachman-Diamond syndrome is an autosomal recessive disorder characterized by short stature, pancreatic exocrine deficiency, and moderate neutropenia.<sup>48,49</sup> Anemia and thrombocytopenia occur in about half of the patients. Over 90% of patients have mutations in the Shwachman-Bodian-Diamond syndrome (*SBDS*) gene. The *SBDS* gene, located in the 7q11 region of chromosome 7, encodes a protein thought to be involved in RNA metabolism. Cytogenetic abnormalities may develop, particularly involving chromosome 7. Approximately 20% of patients can be expected to develop leukemia/myelodysplasia over the course of 20 years.<sup>49</sup>

Patients with Chediak-Higashi syndrome have cutaneous and ocular hypopigmentation, the presence of large abnormal granules in all granule-containing cells, frequent pyogenic infections, and the propensity to develop a late lymphoma-like accelerated phase. Neutropenia is usually mild, and the neutrophils are dysfunctional.<sup>50</sup> Mutations occur in the lysosomal tracking regulator gene, probably associated with protein transport to and from the lysosome.<sup>51</sup>

The p14 deficiency syndrome is characterized by severe neutropenia, partial albinism, short stature, and recurrent infections. The defect is a mutation in the gene encoding p14, an adaptor protein involved in mitogen-activated protein kinase signaling.<sup>52</sup>

Cyclic neutropenia is an autosomal dominant or sporadically occurring disorder characterized by regular oscillations of leukocytes, platelets, and reticulocytes, with predictable severe neutropenia occurring every 21 days.<sup>53</sup> These patients all have mutations in ELA2,<sup>54</sup> the likely mechanism being alterations in the rate of neutrophil apoptosis.

Neutropenia can also be seen in a number of broader metabolic or immunologic syndromes, some examples of which are listed in Table 14-1. Severe neutropenia and neutrophil dysfunction frequently accompany type 1b glycogen storage disease, an autosomal recessive disorder attributable to mutations in the glucose-6-phosphate-translocase gene.<sup>55</sup> Reticular dysgenesis is a neonatal disorder characterized by thymic aplasia and lack of lymphoid or myeloid development.<sup>56</sup> Patients are prone to serious bacterial and viral infection, and death occurs in early infancy. Dyskeratosis congenita is a rare disorder with dystrophic abnormalities in nails, skin, and mucous membranes, neutropenia, and an increased incidence of malignancy. Inheritance

can be X-linked or autosomal dominant or recessive. The gene involved in the X-linked variety has been mapped to Xq28 and is thought to be involved in cell cycle function.<sup>57</sup> Barth syndrome is X-linked and characterized by neutropenia, short stature, and cardiac and skeletal muscle myopathies. The genetic defect is a mutation in the gene that encodes tafazzin, a protein expressed in cardiac and skeletal muscle.<sup>58</sup> Griscelli syndrome is characterized by immune deficiency, partial albinism, neurologic abnormalities, lymphohistiocytosis, thrombocytopenia, and neutropenia. Various subtypes exist; the one related to hematologic abnormalities is associated with mutations in the *Rab27a* gene, which encodes a guanosine triphosphate (GTP)-binding protein.<sup>59</sup>

Chronic idiopathic neutropenia may be congenital or acquired and is characterized by absence of other hematologic findings, splenomegaly, or chromosomal abnormalities.<sup>60</sup> The marrow appears normal except for reduced mature neutrophils in certain patients. Clinical problems with infection tend to be more severe in patients with the lowest neutrophil counts and the fewest neutrophil precursors in the marrow. However, susceptibility to infection is best judged by the patient's experience, because some patients with very low counts have little clinical difficulty. Progression to malignancy or more serious hematologic disease is unusual.

Isolated neutropenia can be seen with deficiencies of folic acid, vitamin B<sub>12</sub>, or copper, although these disorders are more often accompanied by other hematologic abnormalities. Pure white cell aplasia is a rare disorder characterized by absence of myeloid cells in the marrow, attributed to autoimmune suppression of neutrophil production.<sup>61</sup>

Immune neutropenia and some neutropenias associated with splenomegaly are probably kinetically attributable to increased neutrophil turnover. Alloimmune neutropenia is a well described but uncommon disorder caused by the transplacental transfer of maternal antibody to neonatal HNA antigens. The clinical course is usually benign, and the disorder is transient, resolving after maternal antibody is metabolized. Autoimmune neutropenia is a poorly characterized syndrome, usually defined by the ability to detect an antibody directed toward a neutrophil antigen, often in the HNA system. In most cases, the clinical picture is similar to that of chronic idiopathic neutropenia.<sup>62</sup> Neutropenia is also occasionally a feature of immune-mediated diseases such as systemic lupus erythematosus and Sjögren syndrome.<sup>63,64</sup> The neutropenia of Felty syndrome (rheumatoid arthritis, splenomegaly, and neutropenia) is most likely attributable to a marrow proliferative defect in concert with reduced blood neutrophil survival caused by immune complexes produced in the spleen.<sup>65</sup>

Increased margination may be seen in patients with splenomegaly or inflammation<sup>21,66-68</sup> and mild neutropenia may result. Whether the increased margination seen with inflammation is a generalized phenomenon or occurs only in the small vessels in the area of inflammation is not known.

Mild to moderate neutropenia can occur in association with a variety of bacterial, viral, parasitic, or rickettsial infections. The mechanisms are poorly understood but probably involve a

combination of marrow suppression, increased adherence to endothelial cells, increased utilization, and splenic sequestration. In the face of overwhelming sepsis, neutropenia may be severe, reflecting increased utilization and exhaustion of the marrow reserves.

### Management of Neutropenia

The principal complication of moderate to severe neutropenia is the increased incidence of infection. Febrile neutropenic patients should be carefully evaluated to identify infection and treated initially with broad-spectrum antibiotics. Once an offending organism is identified, the antibiotic regimen can be adjusted accordingly. Prophylactic use of antibiotics may be associated with the emergence of resistant organisms, gastrointestinal complaints, and allergic reactions; nevertheless, their use may be appropriate in patients with frequent infections.

G-CSF therapy is often administered for severe neutropenia caused by an idiosyncratic drug reaction, even though most studies have not been able to demonstrate a survival advantage. Recent studies have suggested that treatment with G-CSF is effective in reducing the rate of infectious morbidity and mortality only in patients with neutrophil counts of less than 100/ $\mu$ L.<sup>41</sup>

G-CSF has proven very successful in treatment of the congenital neutropenias. Dale et al<sup>69</sup> reported a randomized controlled trial of daily G-CSF treatment in 123 patients with recurrent infections and severe chronic neutropenia (absolute neutrophil count less than 500/ $\mu$ L). Patients were randomly assigned to G-CSF treatment or 4 months of observation; those in the observation arm were subsequently treated with G-CSF. Ninety-three percent of patients responded. On therapy the mean blood neutrophil count was 1500/ $\mu$ L, marrow examination revealed an increased proportion of mature neutrophils, the incidence and duration of infectious episodes were decreased 50%, and the duration of antibiotic use decreased 70%. More recently, the Severe Chronic Neutropenia International Registry reported data on 853 patients followed since 1994, most of whom were treated with daily or every-other-day G-CSF.<sup>45,47,70</sup> Over 95% of patients responded to therapy; success was defined as achieving a blood neutrophil count in excess of 1000/ $\mu$ L. Mean blood neutrophil counts increased from 300/ $\mu$ L to 3700/ $\mu$ L over the course of the first year; these responses were for the most part long lasting with continued drug treatment. G-CSF has also been shown to be effective in patients with Shwachman-Diamond syndrome,<sup>49</sup> myelokathexis,<sup>71</sup> Felty syndrome,<sup>72</sup> autoimmune neutropenia,<sup>73</sup> and glycogen storage disease type 1b.<sup>74</sup> Myelodysplasia and acute leukemia have developed in 11.5% of patients with severe congenital neutropenia or Shwachman-Diamond syndrome (but not in other disorders listed in Table 14-1) followed over 5 to 6 years on G-CSF therapy.<sup>45</sup> The annual incidence increases with the duration of therapy and with higher doses of G-CSF. Leukemic conversion is associated with the development of other genetic abnormalities such as monosomy 7, ras mutation, or mutation of the G-CSF receptor (resulting in a truncated C-terminal cytoplasmic region). Because genetic instability and leukemic transformation were known to be part of these disorders before the



Table 14-2. Mechanisms and Causes of Neutrophilia

| Mechanism                      | Causes                                                                                                                                                                                                                                                                                     |                                                                                                                                                  |
|--------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------|
|                                | Acute Neutrophilia                                                                                                                                                                                                                                                                         | Chronic Neutrophilia                                                                                                                             |
| Decreased margination          | <ul style="list-style-type: none"><li>• Stress<ul style="list-style-type: none"><li>– Hypoxia</li><li>– Intoxication</li></ul></li><li>• Exercise</li><li>• Epinephrine</li></ul>                                                                                                          |                                                                                                                                                  |
| Mobilization from marrow       | <ul style="list-style-type: none"><li>• Infection</li><li>• Inflammation</li><li>• Stress<ul style="list-style-type: none"><li>– Hypoxia</li><li>– Intoxication</li></ul></li><li>• Prolonged exercise</li><li>• Granulocyte colony-stimulating factor</li><li>• Corticosteroids</li></ul> |                                                                                                                                                  |
| Prolonged survival             | <ul style="list-style-type: none"><li>• Corticosteroids</li><li>• Granulocyte colony-stimulating factor</li></ul>                                                                                                                                                                          | <ul style="list-style-type: none"><li>• Myeloproliferative disorders</li><li>• Corticosteroids</li><li>• Leukocyte adhesion deficiency</li></ul> |
| Increased marrow proliferation | <ul style="list-style-type: none"><li>• Rebound from neutropenia</li></ul>                                                                                                                                                                                                                 | <ul style="list-style-type: none"><li>• Infection</li><li>• Inflammation</li><li>• Myeloproliferative disorders</li></ul>                        |

advent of G-CSF therapy, it is not clear whether the increased incidence seen with therapy is an effect of G-CSF or merely reflects the fact that the patients live longer on therapy.<sup>45</sup>

Patients with febrile neutropenia may also benefit from G-CSF therapy. A randomized controlled study compared G-CSF/antibiotic therapy with that of antibiotics alone and demonstrated that patients receiving G-CSF had shorter duration of neutropenia and fever and a reduced likelihood of prolonged hospitalization.<sup>75</sup> The greatest benefit appeared to be for patients with documented infection and very severe neutropenia (neutrophil counts less than 100/ $\mu$ L).

Neutrophilia

Neutrophilia, defined as a blood neutrophil count in excess of 7500/ $\mu$ L, may be either acute or chronic (Table 14-2). Unlike the case with neutropenia, an elevated level of mature blood neutrophils usually has no clinical implications in itself; rather, it serves to alert the clinician to underlying events.

A shift of cells from the marginal pool to the circulating pool, resulting in neutrophilia, may be seen as an effect of epinephrine, in situations of acute stress, or with vigorous exercise. The effect is a rapid one, usually occurring within minutes, and is short lived, the neutrophil count rapidly returning to baseline when

the stimulus is removed. The neutrophil count usually will no more than double, and immature neutrophils are not seen.

Acute neutrophilia may also occur with mobilization of the marrow neutrophil reserves, most commonly as a result of infection or inflammation. The extent of the storage pool shift under these conditions depends on the intensity of the stimulus and will be reflected in the magnitude of the neutrophilia and in the degree to which immature forms are seen in the blood.

Chronic neutrophilia most commonly represents the marrow’s normal reaction to infection or inflammation. The marrow proliferative pool expands, increasing marrow neutrophil production several times over baseline. Chronic neutrophilia is also a feature of the myeloproliferative diseases, where marrow neutrophil production is autonomous.<sup>76</sup> These disorders can usually be distinguished from the more common benign reactive neutrophilias by the presence of additional features such as erythrocytosis, thrombocytosis, eosinophilia, basophilia, a more pronounced shift to the left (more immaturity), splenomegaly, a low leukocyte alkaline phosphatase (in chronic myelocytic leukemia), chromosomal abnormalities, or the presence of the *BCR* gene rearrangement.

Chronic neutrophilia is also caused by reduced migration of neutrophils from the blood to the tissues. This mechanism is responsible for the mild to moderate neutrophilia seen with chronic corticosteroid therapy.<sup>77,78</sup>

Patients with reactive leukocytosis occasionally present with leukocyte profiles that are indistinguishable from that of leukemia—so-called leukemoid reactions. Usually there is marked leukocytosis (more than 50,000 cells/ $\mu$ L) with immature forms present, and the confusion is with the myeloproliferative syndromes (most commonly chronic myelocytic leukemia), although acute leukemias may be simulated. The absence of the characteristic features of the myeloproliferative disorders listed above (eg, basophilia, chromosomal markers) is often helpful in the differential diagnosis. However, the diagnosis of a leukemoid reaction can be considered certain only if the leukocyte profile returns to normal when the underlying infectious or inflammatory disorder resolves.

Disclaimer

The author has disclosed no conflicts of interest.

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# 15 Neutrophil Collection and Transfusion

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Life-threatening infections with bacteria, yeast, and fungus continue to be a consequence of severe neutropenia (fewer than 500/ $\mu$ L blood neutrophils) and disorders of neutrophil (PMN) dysfunction. The most frequent situation is neutropenic fever and infection with yeast or fungus during either hematopoietic progenitor cell (HPC) transplantation or intense chemotherapy for hematologic malignant disease. Neutropenic infections cause considerable morbidity, occasionally are fatal, and add considerable cost to the treatment of these patients.

Previous attempts to prevent infection in severely neutropenic patients by transfusing PMN concentrates—commonly called prophylactic granulocyte transfusions (GTXs)—achieved only questionable success. Although rates of certain infections were significantly reduced with prophylactic GTX compared to antibiotics only, many adverse effects including pulmonary infiltrates and cytomegalovirus infection were reported, and GTXs were expensive. Similarly, use of therapeutic GTX to resolve existing infections has not gained broad acceptance, despite many reports documenting benefit.<sup>1,2</sup> This lack of enthusiasm for GTX can be explained by the continuing development of effective antimicrobial drugs to prevent and manage infection, by the availability of recombinant hematopoietic growth factors to quicken PMN recovery, by preference for peripheral blood HPC (HPC-A) transfusions over marrow-derived HPC (HPC-M) transplantation to hasten patient recovery from myelotoxic therapy and shorten the period of severe neutropenia, and by lack of familiarity by some physicians with the markedly improved PMN concentrates now available for transfusion.

Historically, PMN concentrates, collected for transfusion from unstimulated donors or those stimulated only with corticosteroids, contained woefully inadequate numbers of PMNs. Currently, much larger numbers of PMNs can be collected from healthy donors by means of granulocyte colony-stimulating

factor (G-CSF) plus corticosteroid marrow stimulation followed by large-volume leukapheresis.<sup>2</sup> This chapter discusses the current technology of PMN collection and provides a critical assessment of the potential for use of therapeutic and prophylactic GTX.

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## Collection of Neutrophils for Transfusion

### Donor Marrow Stimulation

A limitation of GTX has been the inability to transfuse satisfactory numbers of adequately functioning neutrophils. To ensure adequate numbers and quality of PMNs for transfusion, PMNs must be collected from optimally stimulated donors by automated leukapheresis using an erythrocyte sedimenting agent, such as hydroxyethyl starch (HES).<sup>2</sup> Under the stress of a severe bacterial infection, the marrow of an otherwise healthy adult will produce between  $10^{11}$  and  $10^{12}$  PMNs in 24 hours. Granulocyte (PMN) concentrates collected from healthy donors who are not stimulated with corticosteroids or G-CSF will contain between 0.2 and  $0.8 \times 10^{10}$  PMNs—about 1% of a healthy marrow's output. Hence, donor stimulation to increase the blood PMN count is mandatory to achieve even a hope of a reasonable PMN dose per GTX.

Donor stimulation with properly timed corticosteroids ( $\geq 4$  hours before leukapheresis) will increase the yield to about  $2 \times 10^{10}$  PMNs.<sup>3</sup> Stimulation with G-CSF, alone or in combination with corticosteroids, will produce higher but variable PMN yields that vary with G-CSF dose and schedule of administration. Currently, PMN donors are optimally stimulated using 300 to 480  $\mu$ g G-CSF given subcutaneously, plus 8 mg dexamethasone taken orally, approximately 12 hours before beginning leukapheresis.<sup>4</sup> Yields of 4 to  $8 \times 10^{10}$  PMNs are achieved regularly, and posttransfusion blood PMN counts in the patient frequently increase to 1000 to 3000/ $\mu$ L, with PMNs detected in the bloodstream for several hours after GTX (often  $>24$  hours).

Adverse effects following G-CSF and dexamethasone are frequent. Most donors experience minor pain in muscle, bone, or head that is readily relieved with acetaminophen or ibuprofen.<sup>5</sup> Donors given G-CSF for PMN collection have no long-term adverse

effects, and it is unusual that they refuse to donate again.<sup>6</sup> A recent report suggested that adrenal corticosteroids might cause posterior subcapsular cataracts in PMN donors.<sup>7</sup> Although this report appeared not to be confirmed by statistically significant findings in a study of larger numbers of PMN donors,<sup>8</sup> many trends—all suggesting possible adverse effects of corticosteroids—were worrisome in the results.<sup>9</sup> Thus, questions of potential donor risk still remain, and it seems logical to include this cautionary information in donor consent forms or to stimulate PMN donors using G-CSF without corticosteroids—accepting that PMN yields will be decreased by about 25%—until the issue is resolved.

Although they are known to alter PMN function, G-CSF and corticosteroids such as dexamethasone have relatively minor effects at the doses administered to donors for PMN collection.<sup>5</sup> The functional properties of PMNs collected from donors stimulated with G-CSF do not deviate greatly from those of normal PMNs and cause no unusual reactions in recipients when transfused. After transfusion, PMNs from G-CSF-stimulated donors have long intravascular survival times explained by multiple factors, including shift of young PMNs from the storage compartment of donor marrow into the bloodstream for collection, an alteration in expression of several membrane proteins on donor PMNs associated with neutrophil adherence to vascular endothelium and egress from the circulation into the tissues, and possibly by specific anti-apoptotic effects of G-CSF on PMNs.<sup>5</sup> Although it has been suggested that G-CSF-mobilized PMNs might exhibit decreased migration into tissue sites because of their prolonged intravascular circulation, studies with transfused PMNs indicate that they migrate satisfactorily into areas of inflammation and infection.<sup>6,10</sup>

The ability to collect and store PMNs differs when donors are stimulated only once during a course of GTX therapy compared with when they serve as a dedicated donor for the recipient and are stimulated repeatedly on a daily or every-other-day schedule for GTX. PMNs collected after several days of G-CSF stimulation are qualitatively different from PMNs collected after a single dose of G-CSF.<sup>5</sup> They are younger, exhibit increased metabolic activity and different surface markers, may possess enhanced antifungal properties, and do not have the same separation characteristics during centrifugation leukapheresis. Because the functional properties and possibly the efficacy and toxicity of PMNs may differ when collected under conditions of single vs repeated G-CSF and corticosteroid stimulation, additional studies are needed to define optimal donor stimulation and GTX therapy.

### Hydroxyethyl Starch

Use of an erythrocyte-sedimenting agent, such as HES, during centrifugation leukapheresis is mandatory. The optimal formulation of HES to be used for PMN collection is controversial. In an uncontrolled multicenter trial, pentastarch appeared to be an efficacious and safe erythrocyte-sedimenting agent for use during centrifugation leukapheresis because PMN concentrates, prepared by centrifugation leukapheresis techniques with pentastarch in four cytopheresis centers, were found to contain quantities of PMNs comparable with concentrates prepared previously

with hetastarch at participating centers.<sup>3</sup> Later, the efficacy of pentastarch for PMN collection was challenged by a study<sup>11</sup> in which steroid-stimulated donors underwent paired PMN collections, separated by 2 weeks to 7 months, during which they received 500 mL of either 10% pentastarch or 6% hetastarch during centrifugation leukapheresis (7 L donor blood processed at a 1:13 starch:donor blood ratio). In 92% of the donors, hetastarch procedures were more efficient. The PMN yield per collection was  $2.3 \pm 0.7 \times 10^{10}$  with hetastarch vs  $1.4 \pm 0.076 \times 10^{10}$  with pentastarch.

It is unclear why pentastarch performed so poorly in the later study<sup>11</sup> compared with its performance in the initial multicenter trial.<sup>3</sup> Until the issue is resolved, it is prudent for each center preparing PMN concentrates to perform continuing quality assessment of its own leukapheresis program. The average PMN yield obtained by means of processing 10 L of donor blood after corticosteroid stimulation *alone* and use either of pentastarch or a hetastarch at a 1:13 starch:donor blood ratio should be between  $1.5$  and  $2.5 \times 10^{10}$ . After G-CSF stimulation plus dexamethasone, the PMN yield should be between  $4$  and  $8 \times 10^{10}$ . If these yields are achieved equally with pentastarch or hetastarch, it seems reasonable to select pentastarch because of its better safety profile—particularly if donors experience repeated leukapheresis at short intervals. Pentastarch is more rapidly eliminated from the bloodstream than is hetastarch and avoids the problem, with repeated leukapheresis, in which hetastarch blood levels accumulate and increase in a staircase manner.<sup>12</sup> Pentastarch exerts lesser effects on coagulation than does hetastarch.<sup>13</sup> If PMN yields are not satisfactory with pentastarch, as is often the case, pentastarch should be replaced by hetastarch.

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## Neutrophil Transfusion in Clinical Medicine

### Historical Overview of Therapeutic Granulocyte Transfusion

To obtain a historical perspective regarding the efficacy of therapeutic GTX (ie, collected without donor G-CSF), seven controlled studies are analyzed briefly.<sup>14-20</sup> In these seven studies, the response of infected neutropenic patients to treatment with GTX plus antibiotics (study group) was compared with that of comparable patients given antibiotics alone (control group) evaluated concurrently. The design, size, and results of these seven studies are presented in Tables 15-1 and 15-2. Three of the seven studies reported a significant overall benefit for GTX.<sup>17-19</sup> In two additional studies,<sup>14,16</sup> overall success was not demonstrated for GTX, but certain subgroups of patients were found to benefit significantly. Thus, some measure of success for GTX was evident in five of the seven controlled studies (Table 15-1). However, this success was counterbalanced by four studies that reported negative results in some respect—two with negative overall results<sup>15,20</sup> and two with negative results for some types of patients.<sup>14,16</sup>

An explanation for these inconsistent results is evident on critical analysis of the adequacy of GTX support (Table 15-2).

Patients in the three successful trials received relatively high doses of PMNs (generally  $\geq 1.7 \times 10^{10}$ /day), and donors were selected to be both erythrocyte and leukocyte compatible.<sup>17,19</sup> By contrast, the four controlled studies reporting overall or partial negative results can legitimately be criticized. Two of the four studies with negative findings used PMNs collected by filtration leukapheresis for some patients.<sup>14,16</sup> It is now established that such PMNs are defective, and they are no longer transfused. In the negative studies using PMNs collected by centrifugation leukapheresis,<sup>14,16,20</sup> the dose was extremely low ( $0.41$  to  $0.56 \times 10^{10}$  per GTX). As another factor, investigators in two of the four negative studies<sup>14,20</sup> made no provision for the possibility of leukocyte alloimmunization, as donors were selected solely on the basis of erythrocyte compatibility. Finally, control subjects responded particularly well to antibiotics alone in three of the four negative studies,<sup>14,15,20</sup> suggesting that these patients fared relatively well with conventional treatment alone, and it was not possible to document additional benefit by adding GTX.

The results of the seven controlled GTX trials have been analyzed by formal meta-analysis,<sup>21</sup> and conclusions were that the low doses of PMNs transfused and the relatively high survival rate of the nontransfused control subjects were primarily responsible for the differing success rates of these studies. In clinical

settings in which the survival rate of nontransfused control subjects was low,<sup>16-19</sup> study subjects benefited from receiving adequate doses of GTX—prompting the authors to suggest that severely neutropenic patients with life-threatening infections should be considered for GTX given in high doses.<sup>21</sup>

### Historical Overview of Prophylactic Granulocyte Transfusion

Based on historical reports, prophylactic GTXs were of marginal value. Some measure of success was found in seven of 12 studies.<sup>22-28</sup> Five studies failed to show a benefit for prophylactic GTXs.<sup>29-33</sup> In none of these five negative studies were large numbers of PMNs obtained from matched donors and transfused daily. In a situation analogous to that for the negative therapeutic GTX trials, the failure of prophylactic GTX might be explained, as least in part, by inadequate transfusions.

In 1997, data from eight of the 12 controlled trials of prophylactic GTX were analyzed quantitatively by means of formal meta-analysis.<sup>34</sup> The findings of the meta-analysis confirmed that variability in the dose of PMNs transfused, inconsistent attempts to provide leukocyte-compatible GTX, and the varying duration of severe neutropenia in different patient groups were primarily responsible for the differing success rates in the reported controlled trials. It was recommended that high doses of compatible PMNs be transfused, if future trials are conducted.<sup>34</sup>

### Assessment of Modern Granulocyte Transfusion Therapeutic Granulocyte Transfusions

A modern therapeutic GTX is defined as a transfusion in which PMNs are obtained from donors stimulated with G-CSF with or without corticosteroids and collected by means of centrifugation leukapheresis with an erythrocyte-sedimenting agent during the processing of relatively large volumes of donor blood. Specifically, the requirements of an ideal PMN collection should include 1) at least 300  $\mu$ g G-CSF given subcutaneously plus 8 mg dexamethasone given orally to the donor approximately 12 hours before leukapheresis is begun, 2) pentastarch or, preferably, hetastarch infused throughout the leukapheresis procedure at a ratio

**Table 15-1.** Historical Controlled Trials of Therapeutic Granulocyte Transfusions

| Investigators                   | Success | Survival    |     |               |     |
|---------------------------------|---------|-------------|-----|---------------|-----|
|                                 |         | Study Group |     | Control Group |     |
|                                 |         | N           | (%) | N             | (%) |
| Higby et al <sup>18</sup>       | Yes     | 17          | 76  | 19            | 26  |
| Volger and Winton <sup>19</sup> | Yes     | 17          | 59  | 13            | 15  |
| Herzig et al <sup>17</sup>      | Yes     | 13          | 75  | 14            | 36  |
| Alavi et al <sup>14</sup>       | Partial | 12          | 82  | 19            | 62  |
| Graw et al <sup>16</sup>        | Partial | 39          | 46  | 37            | 30  |
| Winston et al <sup>20</sup>     | No      | 48          | 63  | 47            | 72  |
| Fortuny et al <sup>15</sup>     | No      | 17          | 78  | 22            | 80  |

**Table 15-2.** Design of Historical Therapeutic Granulocyte Transfusion Controlled Trials

| Investigators                   | Randomized | Characteristics of Neutrophil Concentrates |                           |          |          |
|---------------------------------|------------|--------------------------------------------|---------------------------|----------|----------|
|                                 |            | Collection Method                          | Dose ( $\times 10^{10}$ ) | Schedule | HLA/WBC* |
| Higby et al <sup>18</sup>       | Yes        | Filtration                                 | 2.2                       | Daily    | Yes      |
| Vogler and Winton <sup>19</sup> | Yes        | Centrifugation                             | 2.7                       | Daily    | Yes      |
| Herzig et al <sup>17</sup>      | Yes        | Filtration                                 | 1.7                       | Daily    | Yes      |
|                                 |            | Centrifugation                             | 0.4                       | Daily    |          |
| Alavi et al <sup>14</sup>       | Yes        | Filtration                                 | 5.9                       | Daily    | No       |
| Graw et al <sup>16</sup>        | No         | Filtration                                 | 2.0                       | Daily    | Yes      |
|                                 |            | Centrifugation                             | 0.6                       | Daily    |          |
| Winston et al <sup>20</sup>     | Yes        | Centrifugation                             | 0.5                       | Daily    | No       |
| Fortuny et al <sup>15</sup>     | No         | Centrifugation                             | 0.4                       | Daily    | Yes      |

\*Donors selected to be compatible with recipient by HLA typing (A and B loci matched, at least in part) and/or by leukocyte crossmatching. WBC = white cell.

of 1 part starch to 12 to 14 parts donor blood, and 3) processing of 8 to 10 L of donor blood. The goal should be to transfuse 6 to  $8 \times 10^{10}$  neutrophils per GTX, with a lower limit of  $4 \times 10^{10}$ .

At this time, no randomized clinical trials of modern therapeutic GTX collected after G-CSF and dexamethasone donor stimulation have been reported to establish either the efficacy or the potential toxicity of modern GTX. However, many case reports suggest success. For example, Clark et al<sup>35</sup> and Catalano et al<sup>36</sup> each reported single patients with aplastic anemia, undergoing progenitor cell transplantation, with fungus infections that responded favorably. Ozsahin et al<sup>37</sup> and Bielora et al<sup>38</sup> each reported single patients with chronic granulomatous disease and fungal infections that responded favorably to GTX during the transplantation period. Bielora et al<sup>39</sup> reported a single patient with acute leukemia and sepsis with vancomycin-resistant *Enterococcus* that cleared slowly with GTX. Similarly, Lin et al<sup>40</sup> reported a single patient with multidrug-resistant *Pseudomonas* sepsis who underwent a successful HPC-A transplantation supported by GTX. It is impossible, in these single reports of very complicated patients, to firmly ascribe the good outcome to the GTX.

Studies of larger numbers of infected, neutropenic patients given GTX from G-CSF-stimulated donors are listed by Table 15-3. Even with investigation of larger numbers of patients, the benefits of GTX are unclear because of the lack of concurrent control patients treated with antibiotics alone. Hester et al<sup>41</sup> transfused 15 patients

with hematologic malignancies and infections. PMNs were collected from donors stimulated only with G-CSF and selected without regard for leukocyte compatibility. Although GTXs were successful in most patients, it was not possible to distinguish responses of fungus vs yeast infections. Grigg et al<sup>42</sup> transfused 11 patients. Eight patients had hematologic malignancies and progressive infections—five of the eight undergoing HPC transplantation and three receiving chemotherapy. Three additional patients who were undergoing HPC transplantation had stable fungus infections. PMNs were collected from donors stimulated only with G-CSF and selected without regard for leukocyte compatibility. Success was excellent for bacterial and stable fungus infections, but was quite poor for progressive fungus infections with organ dysfunction—a troubling pattern reported later by others.<sup>6,43</sup> Peters et al<sup>44</sup> transfused 30 patients with hematologic disorders—18 undergoing HPC transplantation. PMNs were collected from donors stimulated with G-CSF or prednisolone and selected without regard for leukocyte compatibility. The exact PMN dose transfused is uncertain, but values from  $0.9 \times 10^{10}$  to  $14.4 \times 10^{10}$  were estimated from data reported. It was impossible to distinguish the success of GTX from G-CSF-stimulated vs prednisolone-stimulated donors. However, the outcome of bacterial infections overall appeared to be superior to that of fungus infections.

Price et al<sup>6</sup> transfused 19 patients (Table 15-3) with hematologic malignancies, 16 of whom had received HPC transplants

**Table 15-3.** Neutropenic Patients Treated with GTX Collected from G-CSF-Stimulated Donors

| Investigators              | PMNs $\times 10^{10}$<br>per Each GTX | Stimulation                                                       | Leukapheresis                                      | Outcomes                                                                                                                                                                                                                                                                                                                          |
|----------------------------|---------------------------------------|-------------------------------------------------------------------|----------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Hester et al <sup>41</sup> | 4.1                                   | G-CSF 5 $\mu$ g/kg                                                | Pentastarch<br>7 L processed                       | 60% (9 of 15) Success with fungus (11 patients) and yeast (4 patients)                                                                                                                                                                                                                                                            |
| Grigg et al <sup>42</sup>  | 5.9*                                  | G-CSF 10 $\mu$ g/kg                                               | Dextran<br>10 L processed                          | 100% (3 of 3) Success with bacterial infection<br>0% (0 of 5) Success with progressive fungus<br>67% (2 of 3) Success with stable fungus                                                                                                                                                                                          |
| Peters et al <sup>44</sup> | 3.5*                                  | G-CSF 5 $\mu$ g/kg<br>or prednisolone                             | Hetastarch<br>6.4 L processed                      | 82% (14 of 17) Success with bacterial infection<br>54% (7 of 13) Success with fungal infection                                                                                                                                                                                                                                    |
| Price et al <sup>6</sup>   | 8.2                                   | G-CSF 600 $\mu$ g/kg<br>plus dexamethasone<br>8 mg                | Hetastarch<br>10 L processed                       | 100% (4 of 4) Success with bacterial infection<br>0% (0 of 8) Success with invasive fungus<br>57% (4 of 7) Success with yeast infection                                                                                                                                                                                           |
| Hubel et al <sup>43</sup>  | 4.6-8.1                               | G-CSF 600 $\mu$ g/kg<br>with or without<br>dexamethasone 8 mg     | Hetastarch<br>or pentastarch<br><br>10 L processed | 55% (unrelated donor) Success with bacterial infection<br>75% (family donor) Success with bacterial infection<br>70% (unrelated donor) Success with yeast infection<br>40% (family donor) Success with yeast infection<br>15% (unrelated donor) Success with fungal infection<br>25% (family donor) Success with fungal infection |
| Lee et al <sup>45</sup>    | 5.1-10.6                              | G-CSF 5 $\mu$ g/kg<br>and/or<br>dexamethasone 3 mg/m <sup>2</sup> | Pentastarch<br>6-10 L processed                    | 40% (10 of 25) Success with multiple-organism infections                                                                                                                                                                                                                                                                          |

\*Assumptions made as PMN dose expressed  $\times 10^{10}$  unclear in these reports. PMN dose calculated using values for the total number and differential count of leukocytes collected, and the volume of units collected (Grigg et al). Dose calculated that would be given to a 70-kg recipient for Peters et al. GTX = granulocyte transfusion; G-CSF = granulocyte colony-stimulating factor; PMNs = granulocytes.

and three who were scheduled for transplantation. PMNs were collected from donors stimulated with G-CSF and dexamethasone. Although donors were selected without regard for leukocyte compatibility, recipients were documented not to exhibit evidence of leukocyte alloimmunization at study entry. Bacterial infections responded well and yeast infections responded modestly. Despite very high PMN doses, success for invasive fungus infections was dismal. Hübel et al<sup>43</sup> expanded the study of Price et al<sup>6</sup> to a total of 74 patients receiving HPC transplants. Comparisons were made to historical control patients ( $n = 74$ ) who did not receive GTX. PMNs were collected either from unrelated donors given G-CSF alone or in combination with dexamethasone, or from family members given G-CSF only. Hetastarch was used for single leukapheresis procedures, and pentastarch was used when donors experienced repeated leukapheresis procedures. Comparative success in nontransfused historical control patients was approximately 90% for bacterial, 40% for yeast, and 30% for fungal/mold infections—values similar to those achieved by GTX recipients. Nontransfused controls actually had better success than PMN recipients for bacterial infections ( $p = 0.04$ ). As a potential adverse effect, allogeneic transplant recipients given GTX from unrelated donors experienced significantly more ( $p = 0.04$ ) Grade IV graft-vs-host disease (GVHD) than controls who were not given GTX. However, relatively few patients were assessed for this endpoint, and firm conclusions are not warranted.

Lee et al<sup>45</sup> transfused 25 patients with hematologic malignancies, many of whom were infected with multiple organisms. Thus, the outcome of infections with specific types of individual organisms could not be determined. PMNs were collected from donors stimulated with G-CSF alone (66% of donors), G-CSF plus dexamethasone (25% of donors), or dexamethasone alone (8% of donors). Of patients with sepsis, 50% (two of four) responded favorably, and 38% (8 of 21) of patients with progressive localized infections responded favorably.

Two studies reported results in ways that did not lend themselves to tabulation, and they will be briefly described. Illerhaus et al<sup>46</sup> transfused 42 neutropenic patients with GTX collected from donors stimulated with  $5\mu\text{g/kg}$  G-CSF. Eighteen of the patients had severe infections with a variety of organisms and each received a median of three GTX each containing a median of  $2.6 \times 10^{10}$  leukocytes. Of these 18 patients, eight (44%) improved, four (22%) stabilized, and six (33%) deteriorated—with four of these last six dying of pulmonary aspergillosis. Rutella et al<sup>47</sup> transfused 20 patients with hematologic malignancies and neutropenic infections. Donors were HLA-identical siblings and each received  $5\mu\text{g/kg}$  G-CSF. Favorable responses were seen in 54% of patients with bacterial and 57% of fungal infections. Underlying cancer status (ie, complete or partial remission achieved) and recovery of blood PMN counts to  $500/\mu\text{L}$  or more were significantly correlated with a favorable response to GTX.

No firm conclusions can be drawn from these larger, but still somewhat anecdotal reports of modern therapeutic GTX for several reasons: 1) no concurrent control subjects were included (ie, randomly assigned to receive antimicrobial drugs, but no

GTX); 2) the number of patients reported, generally, was quite small; and 3) PMN collection methods were variable with a broad range of PMN doses transfused. Based on these preliminary findings, bacterial infections appeared to respond quite well to modern GTX, and relatively mild fungus and yeast infections responded modestly well. However, serious fungus infections with tissue invasion often resisted even the large doses of PMNs transfused with modern GTX.<sup>6,42-46</sup> The precise role of modern therapeutic GTX, collected from donors stimulated with G-CSF plus corticosteroids, in the management of infected, neutropenic, oncology/transplant patients awaits definition by randomized clinical trials.

### Prophylactic Granulocyte Transfusions

The role of modern prophylactic GTX (ie, from G-CSF-stimulated donors) has not been established by definitive clinical trials. However, two factors suggest possible success: 1) rapid recovery from myeloablation, hastened by HPC-A transfusions and treatment of patients with recombinant growth factors, has shortened the period of severe neutropenia to as brief as 1 week; and 2) this relatively brief period of severe neutropenia might literally be eliminated by transfusing large doses of PMNs collected from donors stimulated with G-CSF plus corticosteroids. A few studies have begun to explore this possibility (Table 15-4).

Bensing et al<sup>48</sup> transfused seven allogeneic marrow recipients with PMNs collected from their HLA-identical or syngeneic marrow donors after G-CSF stimulation. Because donors experienced leukapheresis on consecutive days, collection techniques were quite variable in terms of quantities of HES infused and liters of donor blood processed, and the number of PMNs per GTX varied from 0.3 to  $14.4 \times 10^{10}$ . Recipients received an average of 7.6 GTXs while awaiting marrow recovery, and recipients were given  $5\mu\text{g/kg}$  G-CSF daily to maintain a mean blood PMN count of  $950/\mu\text{L}$ , measured 24 hours after transfusion. The goals of this study were to evaluate the feasibility and safety of collecting and transfusing PMNs from G-CSF stimulated donors; patient outcomes were not reported.

Adkins et al<sup>49</sup> transfused 10 allogeneic marrow recipients with PMNs collected from their HLA-matched sibling marrow donors. Leukapheresis was performed on Days 1, 3, and 5 after transplantation, and GTXs were administered on these days. Recipients were given  $7.5\mu\text{g/kg}$  G-CSF every 12 hours until blood PMNs were greater than or equal to  $1500/\mu\text{L}$ . Recipient blood PMNs were maintained at greater than  $500/\mu\text{L}$  throughout the 5 days of posttransplant GTX. By comparison, a historical group of control recipients treated with G-CSF, but no GTX, exhibited mean blood PMN counts less than  $500/\mu\text{L}$  following transplantation. Prophylactic GTX seemed promising in this setting and, perhaps, would have been even more effective (ie, higher recipient blood PMN counts and fewer infections) if given daily. In another study, Adkins et al<sup>50</sup> transfused 23 autologous HPC-A recipients with PMNs collected from first-degree relative donors. Leukapheresis was performed on posttransplant Days 2, 4, 6, and 8, and GTXs were given on those days.



**Table 15-4.** Prophylactic GTX Using PMNs from G-CSF-Stimulated Donors in Hematopoietic Progenitor Cell Recipients

| Investigator                  | PMNs × 10 <sup>10</sup><br>per Each GTX | Stimulation                                    | Leukapheresis                                    | Outcomes                                                                                |
|-------------------------------|-----------------------------------------|------------------------------------------------|--------------------------------------------------|-----------------------------------------------------------------------------------------|
| Bensinger et al <sup>48</sup> | 4.2                                     | G-CSF 3.5-6μg/kg                               | Variable                                         | Not reported                                                                            |
| Adkins et al <sup>49</sup>    | 4.1 (Day 1)                             | G-CSF 5μg/kg × 5<br>days after transplantation | Hetastarch                                       | 60% (6 of 10) afebrile                                                                  |
|                               | 5.1 (Day 3)                             |                                                | 7 L processed                                    | 40% (4 of 10) febrile                                                                   |
|                               | 6.1 (Day 5)                             |                                                | Days 1, 3, and 5                                 | Three culture positive                                                                  |
| Adkins et al <sup>50</sup>    | 5.6 (Day 2)                             | G-CSF 10μg/kg                                  | Hetastarch                                       | Reduction of fever and antibiotics if<br>no leukocyte antibodies                        |
|                               | 7.0 (Day 4)                             |                                                | 7 L processed                                    |                                                                                         |
|                               | 8.5 (Day 6)                             |                                                | Days 2, 4, 6, and 8                              |                                                                                         |
|                               | 9.9 (Day 8)                             |                                                |                                                  |                                                                                         |
| Oza et al <sup>51</sup>       | 5.9 (Days 3 and 5)                      | GCSF 10μg/kg                                   | Hetastarch                                       | Less fever and antibiotics when<br>GTX given; no effect on<br>hospital stay or survival |
|                               | 5.2 (Days 6 and 7)                      |                                                | 7 L processed<br>Days 3 and 6 or<br>Days 5 and 7 |                                                                                         |

GTX = granulocyte transfusion; PMNs = granulocytes; F-CSF = granulocyte colony-stimulating factor.

Recipients were given 5 μg/kg G-CSF daily until the blood PMN count was greater than or equal to 1500/μL. Recipients were studied for the effects of lymphocytotoxic antibodies (ie, leukocyte alloimmunization) on GTX effectiveness. The 15 recipients who did not exhibit lymphocytotoxic antibodies during the 10-day study period experienced a mean of 4.1 febrile days and required 7.3 days of antibiotics. Values for the eight recipients with lymphocytotoxic antibodies were less desirable—6.3 febrile days and 10.5 days of antibiotics. Rates of documented infections were not reported.

In a later report from the same group,<sup>51</sup> 151 donor-recipient pairs, consisting of HLA-matched sibling donors of peripheral blood progenitor cells and the recipients of their progenitor cells, were initially allocated to either a GTX group or a non-GTX group based on ABO matching of red cells. For several reasons, the final allocation was 53 ABO-matched donor-recipient pairs assigned to GTX and 98 pairs assigned to the non-GTX group. PMNs were collected for GTX on either Days 3 and 6 or on Days 5 and 7 after transplantation (different days due to enrollment into two different studies). Thus, recipients received two GTXs on an every-other-day schedule. Recipients also received G-CSF (10 μg/kg/day) beginning 1 day after transplantation and continuing until a blood PMN count was equal to or greater than 1500/μL for 3 days. The percentage of recipients with posttransplant fever was 64% with GTX vs 83% without GTX (*p* = 0.03). The median number of days that intravenous antibiotics were given was 9 with GTX vs 11 without GTX (*p* = 0.03). No significant differences were found for length of hospital stay, 100-day survival, or rates of acute GVHD (Table 15-4).

No firm conclusions can be drawn from these reports of modern prophylactic GTX because few nontransfused control subjects were included (none randomly assigned before the studies); relatively few patients were studied; and most patients were given GTXs every other day, rather than daily—possibly

providing a less than optimal dose of PMNs. Modern prophylactic GTXs appear promising, but their efficacy, potential adverse effects, and economic analysis await definition by randomized clinical trials of sufficient numbers of patients.

**Pediatric Granulocyte Transfusion**

Therapeutic GTXs are prescribed for children with marrow failure and severe neutropenic infections using the same criteria as for adults. In a study by Sachs et al,<sup>52</sup> 27 children with hematologic/oncologic disorders and neutropenic infections received GTXs every other day. PMNs were collected from crossmatch-compatible donors stimulated with G-CSF only. The average dose of PMNs per each GTX was 8 × 10<sup>8</sup> PMN/kg recipient body weight. This corresponds to 5.6 × 10<sup>10</sup> PMNs for a 70-kg adult. GTXs were very successful with 93% of patients able to clear infections—including all with invasive pulmonary aspergillosis infections—and with an 82% survival 1 month later.

Most patients with congenital disorders of PMN dysfunction have adequate numbers of blood PMNs, but they are susceptible to serious infections because their PMNs fail to kill pathogenic microorganisms. Patients with severe forms of PMN dysfunction are relatively rare, and no randomized clinical trials have been reported to establish the efficacy of therapeutic GTX in their management. Firm recommendations about the use of GTX to treat patients cannot be made. However, several patients with chronic granulomatous disease, complicated by progressive life-threatening fungal infections, have been reported to benefit.<sup>53-61</sup> Because of the possibility of alloimmunization to leukocyte and red cell antigens, particularly to the Kell blood group, plus the risk of transfusion-transmitted infections, therapeutic GTXs are recommended only for progressive infections that cannot be controlled with antimicrobial drugs. Because of lifetime problems with infections, prophylactic GTXs are impractical.

Neonates (infants within the first month of life) are another group of patients who may suffer life-threatening bacterial infections caused, at least in part, by PMN dysfunction and neutropenia. Neutropenia must be viewed differently in neonates than in older patients, because in the latter, GTXs are considered usually when the blood PMN count falls to less than 500/ $\mu$ L. By contrast, because normal neonates exhibit a physiologic neutrophilia, absolute blood PMN counts as high as 3000/ $\mu$ L (ie, relative neutropenia due to age) might prompt consideration of GTX in neonates. Six controlled trials (reviewed in Strauss<sup>62</sup>) have assessed the role of GTX in treating neonatal infections. Although four of the six found a significant benefit for GTX, the controlled studies, when assessed by meta-analysis, were insufficiently homogeneous to permit clear recommendations regarding the efficacy of GTX.<sup>21</sup> Thus, the role of therapeutic GTX for neonatal infections is unclear at present and, because they are transfused only rarely at this time, they will not be discussed further.

## Summary and Recommendations

The use of G-CSF with glucocorticoids to stimulate PMN donors has brought GTX therapy into a new era. It is now possible to collect relatively large numbers of PMNs by modern leukapheresis techniques. Because GTXs were successful historically in the management of bacterial infections, even when PMNs were given in low doses, it is prudent to reassess both therapeutic and prophylactic GTX.

To determine whether a need exists for therapeutic GTX, physicians should survey the outcome of neutropenic infections at their own institutions. If these infections respond promptly to antibiotics alone and the survival rate approaches 100%, therapeutic GTX is unnecessary and should not be used, because possible benefits are small and would not outweigh the risks and expense. In contrast, if patients with infection and neutropenia (fewer than 500 PMN/ $\mu$ L) do not respond quickly and completely to antibiotics alone, the addition of therapeutic GTX should be considered along with other modifications of therapy, such as selection of different antibiotics, closer monitoring of antibiotic blood levels, intravenous  $\gamma$ -globulin therapy, and treatment with recombinant myeloid growth factors.

Prophylactic GTX is most likely to be useful in the setting of HPC transplantation. Both autologous and allogeneic HPC-M transplantation have been largely supplanted by HPC-A transfusion—a technique that is convenient and economical, and leads to relatively rapid PMN engraftment. Recovery to a blood PMN count of at least 500/ $\mu$ L occurs usually within 7 to 14 days after HPC-A transfusion. Future HPC-A donors may be stimulated with recombinant cytokines other than G-CSF alone, such as AMD3100 (antagonist of the receptor for stromal-cell-derived factor 1), stem cell factor, or interleukin-7; the period of severe neutropenia may be shortened even more by transfusing these PMNs. Thus, complete elimination of severe neutropenia with a

strategy of transplanting HPC-A followed by prophylactic GTX is a distinct possibility that deserves more study.

Once the decision has been made to provide either therapeutic or prophylactic GTX, PMNs must be collected and transfused optimally as follows:

1. Collect PMNs from allogeneic donors with a goal to transfuse 6 to 8  $\times 10^{10}$  PMNs per GTX with an absolute lower limit of 4  $\times 10^{10}$ . The need to select donors of who are leukocyte compatible with recipients has not been established for modern (high PMN dose) GTX, despite the historical importance of doing so when lower doses of PMNs were transfused. In studies of modern GTX, Price et al<sup>6</sup> found no apparent adverse effects of several leukocyte antibodies (granulocyte agglutinating, granulocyte immunofluorescent, lymphocytotoxic, and lymphocyte immunofluorescent) that became detectable in the recipient's blood during GTX therapy. Adkins et al,<sup>50</sup> however, found the presence of lymphocytotoxic antibodies to be a poor prognostic factor, in general, even though actual donor-recipient incompatibility for individual GTX was rare. Until more data are available, it seems prudent to select donors who are leukocyte compatible (eg, by means of HLA matching or leukocyte crossmatching) whenever easily feasible, but never to delay or deny GTX therapy if selecting donors by these methods is not readily accomplished.
2. Stimulate donor neutrophilia by giving 300 to 600  $\mu$ g G-CSF subcutaneously plus dexamethasone orally 12 hours before beginning leukapheresis. A regimen reported to produce high PMN yields with reasonable adverse effects on the donor is 450  $\mu$ g G-CSF given subcutaneously plus 8 mg dexamethasone given orally 12 hours before leukapheresis.<sup>4</sup>
3. Process 8 to 10 L of donor blood using a continuous-flow, centrifugation blood separator with citrated HES (hetastarch preferred) solution infused throughout the entire collection at a starch:donor blood ratio of 1:13.
4. Transfuse PMNs as soon as possible after collection to minimize storage damage. Data suggest deterioration of PMN function begins within a few hours of storage. Until the efficacy of stored neutrophils from G-CSF-stimulated leukapheresis donors is documented, it seems wise to transfuse within 6 hours or so of collection. This timing may require agreement by the patient's physician to perform infectious disease testing on the donor before actual PMN collection (eg, at the time G-CSF is administered before leukapheresis) or accept a donor who has been tested recently for a previous donation (eg, within approximately 10 days), but has not been tested for the PMN collection in question.
5. Give GTX daily as prophylactic GTX in an attempt to prevent infection during the period of severe neutropenia or as therapeutic GTX either to resolve a documented infection—as evidenced by clearing of tissue lesions, negative cultures, or resolution of fever—or until marrow function recovers to produce adequate numbers of endogenous PMNs. Determining marrow recovery may be difficult because PMNs collected from G-CSF-stimulated and dexamethasone-stimulated donors and transfused at doses of 6 to 8  $\times 10^{10}$  PMNs may elevate the recipient's blood PMN count to more than 1000/ $\mu$ L for more than 24 hours. Accurate

differentiation of transfused PMNs from those produced endogenously is challenging, and marrow recovery must be based on a sustained increase in blood PMN count after GTX is discontinued.

## Disclaimer

The author has disclosed no conflicts of interest

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# 16

## Leukocyte-Reduced Blood Components: Laboratory and Clinical Aspects

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Donor leukocytes present in cellular blood components have been linked to a wide range of complications in transfusion recipients. Both acute and long-term complications can result directly from exposure to donor leukocytes, prompting the adoption of leukocyte reduction as a highly effective and widely practiced prevention strategy. For other complications, the association with donor leukocytes is less certain, and more research is needed before specific transfusion practice can be established. This chapter reviews the current technologies for leukocyte reduction, the scientific basis for adverse effects of exposure to donor leukocytes, and the evidence for leukocyte reduction of cellular components. The reader is also referred to recent reviews of this topic.<sup>1,2</sup>

### Laboratory Aspects

Fresh cellular blood components contain considerable quantities of residual donor leukocytes (Table 16-1). However, during refrigerated storage of Red Blood Cells (RBCs), a substantial proportion of donor leukocytes undergo cellular degeneration and apoptosis, and surface antigens on residual cells change. Thus, refrigerated storage itself may help prevent complications from transfused donor leukocytes to a degree not completely appreciated. In the 1980s, washed or frozen deglycerolized RBCs were used to prevent complications resulting from transfusion of donor leukocytes. During the 1990s, several technologies were introduced to remove or inactivate residual donor leukocytes in blood components. High-performance leukocyte reduction filters are used for either RBCs or whole-blood-derived platelet concentrates. Apheresis platelets can be leukocyte reduced (LR) at the time of collection without the use of filters. Chemical pathogen reduction systems designed specifically for RBCs, platelet concentrates, or plasma are undergoing clinical trials. These chemicals inactivate residual donor leukocytes. The potential

**Table 16-1.** Approximate Residual Number of Leukocytes in Cellular Blood Components

| Component                        | No. of Leukocytes   |
|----------------------------------|---------------------|
| Fresh Whole Blood                | $10^9$              |
| Red Blood Cells (RBCs)           | $10^8$ - $10^9$     |
| Buffy-coat-depleted RBCs         | $10^8$              |
| Washed RBCs                      | $10^7$              |
| Frozen deglycerolized RBCs       | $10^6$ - $10^7$     |
| Whole-blood-derived platelets    | $10^7$ - $10^8$     |
| Apheresis platelets              | $10^6$ - $10^8$     |
| Leukocyte-reduced (LR) RBCs      | $<5 \times 10^6$    |
| LR whole-blood-derived platelets | $<0.83 \times 10^5$ |
| LR apheresis platelets           | $<5 \times 10^6$    |
| Fresh Frozen Plasma              | $<10^4$             |

of these diverse technologies to affect known complications of recipient exposure to donor leukocytes is shown in Table 16-2.

### Technologies for Leukocyte Reduction

#### Centrifugation

Removal of the buffy coat from whole blood during initial component preparation is widely practiced in Canada, Europe, and Japan. In one technique, called the *top-and-bottom method*, whole blood is subjected to centrifugation at relatively high gravity force to compress both platelets and leukocytes into the buffy coat (Fig 16-1). Preparation of the buffy coat component is sensitive to minor changes in centrifugation and separation conditions. Automated devices have been developed to render the process more reproducible. Buffy-coat-reduced RBCs contain approximately 70% to 80% fewer leukocytes than do unmodified RBCs. This advantage is offset to some degree by the loss of platelets and red cells that occurs during the procedure. Buffy coat depletion is sufficient to prevent many febrile nonhemolytic transfusion reactions (FNHTRs) to blood but is not considered sufficient to prevent HLA alloimmunization or cytomegalovirus (CMV) transmission.

**Table 16-2.** Potential Use of Technologies Designed to Reduce the Risk of Complications Attributed to Residual Donor Leukocytes

| Use                                                       | Leukocyte-Reduced Blood | $\gamma$ Irradiation | Ultraviolet-B Irradiation | Chemical Inactivation |
|-----------------------------------------------------------|-------------------------|----------------------|---------------------------|-----------------------|
| Reduce rate of HLA antibody formation                     | Yes                     | No                   | Yes                       | Uncertain             |
| Reduce risk of transmission of leukotropic viruses        | Yes                     | No                   | No                        | Yes                   |
| Reduce risk of FNHTRs due to donor cytokine               | Yes, if prestorage      | Unproven             | Probable                  | Probable              |
| Reduce risk of FNHTRs due to reaction against donor cells | Yes                     | No                   | No                        | Uncertain             |
| Reduce risk of TA-GVHD                                    | Unproven                | Yes                  | Animal studies only       | Animal studies only   |

FNHTR = febrile nonhemolytic transfusion reaction; TA-GVHD = transfusion-associated graft-vs-host disease.

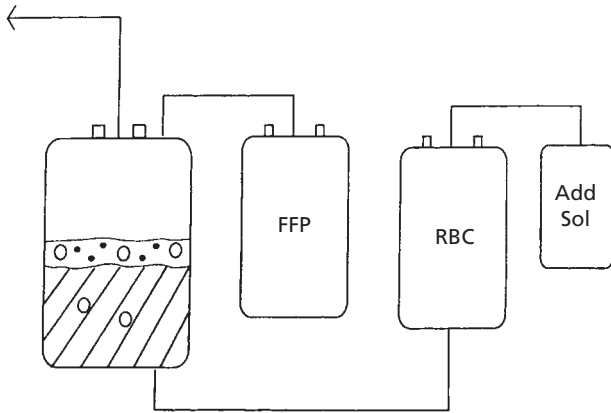


**Figure 16-1.** Preparation of buffy-coat-depleted blood components from whole blood by means of the top-and-bottom method. The primary bag (left) has undergone centrifugation at a high-gravity force. The buffy-coat-containing leukocytes and platelets are between the plasma (open area) and red cells (hatched area). The supernatant plasma is expressed out the top of the primary bag into a satellite bag while the red cells are expressed out the bottom into another satellite bag. The buffy-coat-containing donor white cells and platelets are then pooled with other buffy coats, and the pool is subjected to centrifugation at a lower gravity force than used previously. After this centrifugation, the pooled platelet-rich plasma is expressed out the top of the pooling bag, and the residual cellular elements are discarded. FFP = fresh frozen plasma; RBC = red cells; Add Sol = additive solution.

## Filtration

Filtration has emerged as the most commonly used method of leukocyte reduction. Because of rapid product development and enhancement in this area, the results of earlier clinical studies underestimate the potential clinical efficacy of current filters. Early leukocyte reduction achieved only 90% to 99% (1 to 2  $\log_{10}$ ) leukocyte reduction. Current high-performance leukocyte removal filters can reduce the residual white cell (WBC) content at least 3 logs and typically achieve 4 log or greater removal. As a result, the proportion of filtered units with less than  $10^6$  residual leukocytes has steadily increased to 99% or greater. Until recently, component-specific filters were required for red cell components and platelet components. The filters designed for red cells removed unacceptable numbers of platelets. A whole blood filter has been developed that provides a LR red cell, platelet, and plasma component using a single filter. Whole blood filter

performance appears comparable to existing single component filters and was recently approved in the United States.<sup>3</sup>

## Mechanisms of Filtration

High-performance filters have been specifically designed to remove leukocytes.<sup>4,5</sup> The plastic housing that contains the filtration medium is designed so that blood encounters a large surface area of medium; the volume of blood retained by the filter (hold-up volume) is minimal; and the medium fits tightly enough within the housing so that blood entering the device cannot bypass the filtration medium. Depletion of leukocytes results primarily from barrier retention in which the pore size of the filter medium is large enough to allow passage of red cells and platelets but small enough to impede passage of leukocytes. To achieve the small pore sizes required, manufacturers have used three different fabrication strategies. In one method, polyester is melted and extruded through fine nozzles into a turbulent gas stream at high velocity. In the process, akin to the formation of cotton candy, the polyester is stretched and cooled to form fine threads of microfibers. These fibers are matted together (like teased hair) and compressed to a controlled fiber density. A variation of this technique is used in products made by Pall Corporation (East Hills, NY), Asahi Kasei Medical Company (Tokyo, Japan), and Fresenius AG (Bad Homburg, Germany). An alternative approach (used by Terumo Corporation, Tokyo, Japan) produces coral-reef-like structures of porous polyurethane with an open-cell geometric configuration containing interconnecting channels that necessitate a circuitous path of flow (Fig 16-2).

Polyester fiber filters consist of a series of layers of fiber material. At the upstream end of the filter, the fiber diameter and effective pore size are large (Fig 16-3). As blood passes through the layers of medium, the fiber diameter becomes smaller, and the pore size decreases to approximately 4 microns (Fig 16-4). Red cells, which are more deformable than leukocyte nuclei, can traverse these small pores. Differences in the viscoelastic properties of red cells and leukocytes are particularly pronounced at low temperatures. This accounts for the better performance of red cell leukocyte reduction filters used at refrigerated temperatures. Because synthetic materials are naturally hydrophobic, they do not become “wet” easily in aqueous solutions. As a result, surface tension would prevent blood from flowing through

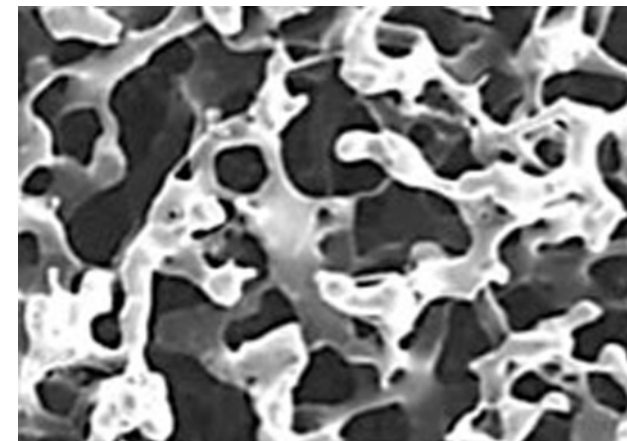


**Figure 16-2.** Electron micrograph of a Terumo filter showing coral-reef-like structures of porous polyurethane with an open-cell geometric configuration containing interconnecting channels that necessitate a circuitous path of flow. (Courtesy Terumo Medical Corporation)

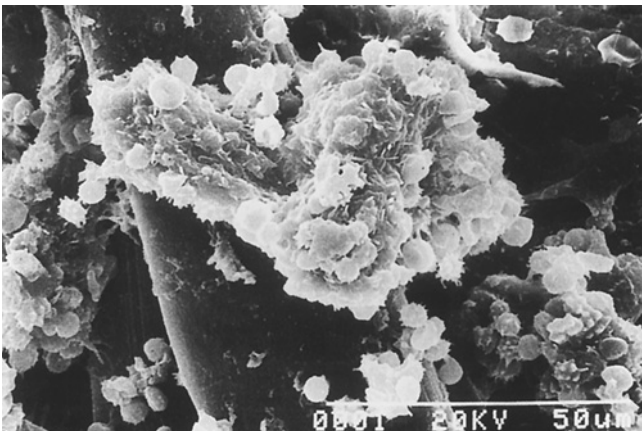


**Figure 16-3.** Electron photomicrograph shows fibers in the coarse filter (upstream layer) of a leukocyte reduction filter designed for RBCs. A microaggregate is present in the center of the figure. The diameter of the pore and the caliber of the fibers relative to individual cells are evident. (Used with permission from Nishimura et al.<sup>6</sup>)

very fine pore spaces under gravity. To overcome this obstacle, manufacturers have modified the surface of the filter medium fibers to increase their “wetability.” The polyurethane matrix in the Terumo filter mechanically traps leukocytes with minimal interaction between the cells or proteins and the filter material.

**Factors Affecting Filter Performance**

Because of the complex physical and biologic forces involved in leukocyte retention by the filter medium, it is not surprising that several factors have been shown to affect the degree of leukocyte reduction obtained (Table 16-3). A primary factor is the capacity of the filter itself. The input load of WBCs to be filtered has a direct relation to the postfiltration WBC content. The temperature of filtration has a dominant effect on the performance

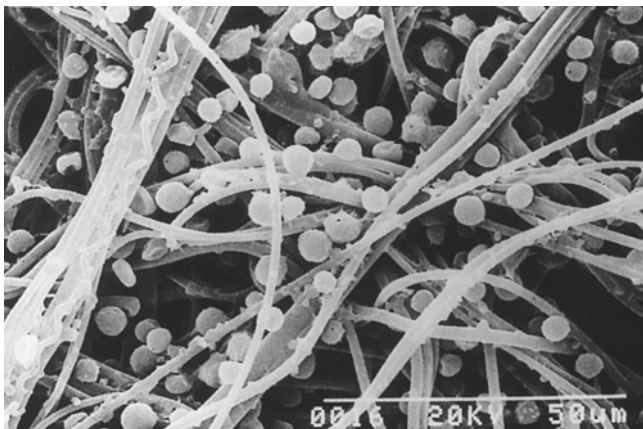


**Figure 16-4.** Electron micrograph shows fibers from the fine filter (downstream layer) of a leukocyte reduction filter designed for RBCs. The caliber of the fibers relative to that of individual cells is evident. (Used with permission from Nishimura et al.<sup>6</sup>)

**Table 16-3.** Factors Affecting Filter Performance

- |                                                                                                                                                                                                                                                                                                                                                                                                      |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <ul style="list-style-type: none"><li>• Capacity of the filter</li><li>• Input number of leukocytes</li><li>• Temperature (viscoelasticity)</li><li>• Flow rate, pressure, priming, rinsing</li><li>• Presence of hemoglobin S</li><li>• Number and function of platelets</li><li>• Holding time between blood collection and filtration</li><li>• Plasma content of cell suspension media</li></ul> |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

of leukocyte filtration of RBCs. Nearly all studies have found that the efficiency of filtration improves when the procedure is conducted at refrigerated temperatures. Beaujean et al<sup>7</sup> and Sirchia et al<sup>8</sup> showed that when RBCs were filtered under conditions that mimicked bedside filtration at room temperature, filtration became less efficient such that most units filtered under bedside conditions over 3 to 4 hours did not meet minimum standards for LR RBCs. Improved performance of filtration at refrigerated temperatures has been observed by other investigators<sup>9,10</sup> and is associated with lower rates of hemolysis of stored RBCs.<sup>11</sup> The dominant effect of temperature on the performance of early filters designed for use with RBCs may have had a subtle effect on the results of the CMV prevention trial conducted by Bowden et al,<sup>12</sup> which was conducted before the temperature effect was realized.

The cellular composition of the blood also affects retention of leukocytes. Blood from donors with sickle cell trait often does not become adequately leukocyte reduced when filtered. Results of studies suggest that approximately 50% of units from donors with hemoglobin AS clog the filter and do not flow. Of the remaining 50%, approximately half of these flow normally but do not undergo adequate leukocyte reduction.<sup>13,14</sup> Preliminary investigation suggests that citrate anticoagulant and low oxygen tension contribute to Hb S polymerization<sup>15</sup> and that sickle trait

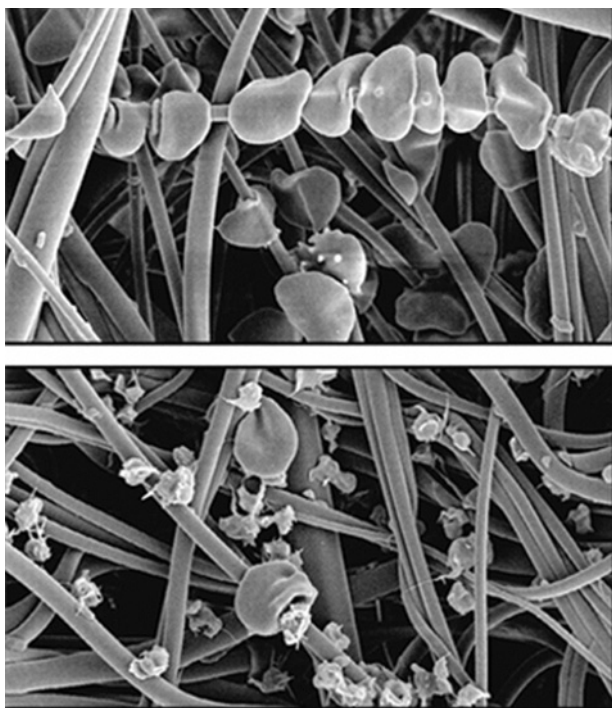


**Figure 16-5.** Electron micrograph demonstrating adherent “sticky” sickle red cells to fibers in a leukocyte reduction filter. (Used with permission from Gorlin and Gudino.<sup>16</sup>)

red cells are far more adhesive to the filter medium<sup>16</sup> (Fig 16-5). As the filter medium is progressively coated with erythrocytes, there is less effective medium area for leukocyte trapping. Other factors that affect the performance of filtration include the protein content of the cell suspension. For example, as little as 5 to 10 mL of plasma added to RBCs suspended in SAG-M additive solution improved the leukocyte removal efficiency of an RC50 filter (Pall Corporation) 10-fold.<sup>17</sup> This observation suggests that adhesive proteins present in plasma contribute to leukocyte retention in some filters. The speed of filtration also has been shown to affect leukocyte reduction. If temperature is held constant, faster flow rates result in decreased performance.<sup>8</sup> This is presumed to result from high shear rates and decreased contact time with the medium. Performing filtration in a controlled laboratory or blood center environment according to the manufacturer’s instructions optimizes the quality of the LR component compared with bedside filtration.

### Low-Leukocyte Apheresis Devices

Red cells collected on apheresis devices cleared by the Food and Drug Administration (FDA) require filtration to achieve the requisite level of leukocyte removal to label the product as leukocyte reduced. However, manufacturers of apheresis devices have modified the instrument to collect platelets with low levels of residual donor leukocytes that require no further filtration to meet standards as LR platelet concentrates. In the United States, three devices—the Spectra LRS (CaridianBCT, Lakewood, CO), Trima

(CaridianBCT), and the Amicus (Fenwal, Lake Zurich, IL)—are approved for collection of LR platelet concentrates without filtration (process leukocyte reduction). These devices achieve a high degree of separation between the donor platelets and the donor leukocytes as a result of several design principles. Flow path geometry, counterflow centrifugation, elutriation, and fluid particle bed separation all are used to separate platelets from leukocytes on the basis of differences in cell mass. Despite advanced design, apheresis systems can fail to collect platelet concentrates that meet standards for being considered leukocyte reduced. Failed collections may result from sudden changes in the donor bleed rate, paused collections that disturb centrifugal separation, or characteristics of the donor blood that interfere with optical sensors on the devices. As in the case of filtration-based systems, some form of process control is required to document that the collected products are leukocyte reduced.

### Process Control of Leukocyte-Reduced Components

To achieve the expected clinical benefits of providing LR blood, facilities need to control the process by which such products are prepared. Process control of leukocyte reduction seeks to establish with a specified statistical confidence that a specified proportion of units conform to the standards for leukocyte reduction. Selecting an appropriate degree of confidence and conformance depend on the expected use of the technology. For example, a greater degree of process control might be applied by facilities that use leukocyte reduction technology as the sole means to prevent transmission of CMV. In contrast, because FNHTRs are not serious events and because prevention of HLA alloimmunization has not been shown to result in improved clinical outcomes, a lower degree of process control may be appropriate if leukocyte reduction is to be used to achieve these two goals. Process control of leukocyte reduction also should document that the prepared products retain an adequate amount of the therapeutic blood cells intended for transfusion. Guidelines for statistical process control of LR blood have been published<sup>18</sup> and draft guidance documents have been issued by the FDA.<sup>19,20</sup>

The concentration of WBCs in a 300-mL LR blood component with  $10^6$  WBCs per unit is only 3.3 WBCs/ $\mu$ L. Because traditional automated cell counters are not accurate at leukocyte concentrations less than 100 WBCs/ $\mu$ L, special techniques are required to accurately count residual WBCs in LR components. The challenges of counting such low levels of leukocytes have been reviewed.<sup>21</sup> All methods depend on counting the cells present in a large volume of blood. Sampling errors and the precision of different methods affect the confidence of the measured result. Several different methods have been published, including cytopspin methods, Nageotte chamber counting, flow cytometry, and counting based on the polymerase chain reaction (PCR). Leukocytes can be stained with either a crystal violet nuclear stain (Turk’s solution), fluorescent dye (propidium iodide), or other vital stains (eg, Plaxan solution). Nageotte chamber counting with crystal violet stain is the most commonly used technique, requires only a light microscope, and has been used for



both RBCs and platelet concentrates. The Nageotte counting chamber has a 50- $\mu$ L bed volume. The sample to be counted is mixed with a red cell or platelet lysing agent and a nuclear stain, allowed to settle undisturbed in the counting chamber, and then examined under 200 $\times$  magnification. The Nageotte counting method has been shown in multicenter evaluations to be accurate to approximately 1 WBC/ $\mu$ L.<sup>22</sup> Because of its simplicity, low cost, and low limit of detection, it is adequate for routine quality control programs. With concentration of a larger volume of blood sample before staining, the lower limit of accurate detection by means of Nageotte counting can be reduced by as much as 100-fold.<sup>23</sup>

Flow cytometry can be used to achieve a lower limit of accurate detection (approximately 0.1 WBC/ $\mu$ L) because a larger volume of sample can be processed. The method requires an expensive instrument and relies on a fluorescent dye that is taken up by leukocyte DNA and emits light when struck by the cytometer laser. With adjustment of the conditions of data acquisition of the cytometer, signals from fluorescent residual leukocytes can be discriminated from those of residual red cells or platelets. Techniques are available for counting WBCs in RBCs and platelet concentrates.<sup>21</sup> Within the range detectable by means of Nageotte counting, the two methods show good correlation.

### **Prestorage, Poststorage, or Bedside Leukocyte Reduction**

Leukocyte reduction by means of filtration can be performed at three different points in time—before storage, after storage but before issue from the blood bank, and at the bedside. Bedside filtration was the first approach to leukocyte reduction and has been shown in clinical studies to be highly effective for the prevention of febrile reactions to RBCs. Although bedside filtration is probably effective for prevention of CMV transmission,<sup>12</sup> one study concluded that it was not effective for prevention of HLA alloimmunization.<sup>24</sup> Bedside filtration has several disadvantages compared with laboratory filtration. These include reduced performance and poor quality control. In addition, there is evidence that febrile reactions to platelet concentrates may be more effectively prevented if leukocytes are removed before storage. Moreover, for some recipients taking angiotensin-converting enzyme (ACE) inhibitors, bedside filtration has been associated with hypotensive transfusion reactions.<sup>25</sup> For these reasons bedside leukocyte reduction is not generally recommended.

Poststorage leukocyte reduction is filtration of components before issue from the blood bank. This approach allows development of an inventory of LR blood. Poststorage filtration is easy to standardize, can be incorporated into the laboratory procedure in a manner similar to that for other manipulations of blood components, and is easily adapted into a program of process control. For whole-blood-derived platelet concentrates, poststorage leukocyte reduction has the disadvantage that cytokines that accumulate during storage are not removed by filtration. For RBCs there is no known disadvantage to poststorage leukocyte reduction.

Prestorage leukocyte reduction during or soon after component preparation is becoming the preferred method of leukocyte reduction and is ideally suited to process control. Opinions differ as to the definition of prestorage leukocyte reduction, but the FDA has suggested that 72 hours be used as the time from collection to filtration. For platelet concentrates, numerous investigators have documented that inflammatory cytokines including interleukin-1 (IL-1), tumor necrosis factor (TNF), and IL-6 can accumulate during storage in some units. The extent of cytokine accumulation correlates with the leukocyte content of the unit and the duration of room temperature storage. For some transfusion recipients, the passively transferred cytokines result in FNHTRs (see later). Prestorage leukocyte reduction of platelet concentrates prevents accumulation of these cytokines. There is speculation that prestorage leukocyte reduction would have additional advantages by preventing transfusion of leukocyte fragments that would otherwise develop during storage. Results of laboratory studies and preclinical animal studies have suggested that leukocyte breakdown may contribute to HLA alloimmunization,<sup>26</sup> release of intracellular viruses,<sup>27</sup> and immunosuppression.<sup>28</sup> However, none of these effects has been documented in clinical trials. Results of a large, randomized, controlled study conducted in Europe with patients undergoing cardiac surgery showed no advantage of prestorage LR RBCs over poststorage LR RBCs for the prevention of postoperative infection, multiple organ failure, or death.<sup>29</sup> Nevertheless, prestorage leukocyte reduction is evolving as the standard method.

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## **Clinical Indications for Leukocyte Reduction**

The clinical indications for the use of LR blood components continue to evolve. New higher-performance filters, the perception of clinical benefit, and the decreasing availability of non-LR blood in many countries have resulted in greater use of LR components. According to a nationwide survey of 2006 data,<sup>30</sup> 72% of RBCs collected in the United States were leukocyte reduced, of which 98% were prestorage leukocyte reduced. Among platelet components, leukocyte reduction was performed on nearly all apheresis platelets and 40% of whole-blood-derived platelets. There has been considerable debate concerning whether all cellular blood components should undergo leukocyte reduction (discussed at the end of the chapter). Evidence-based clinical indications for the use of LR blood have been proposed<sup>1</sup> and are listed in Table 16-4.

### **Reduction in Febrile Nonhemolytic Transfusion Reactions**

Reduction in the rate of recurrent FNHTRs to RBCs was among the earliest applications of leukocyte reduction. Washed RBCs, frozen deglycerolized RBCs, and RBCs filtered through micro-aggregate blood filters all were effective at preventing FNHTRs. This success was based in large part on the fact that FNHTRs may be prevented with blood containing fewer than  $5 \times 10^8$  WBCs

**Table 16-4.** Clinical Indications for Leukocyte-Reduced Blood Components**Established Indications**

- Reduce frequency of recurrent febrile nonhemolytic transfusion reactions to Red Blood Cells (RBCs)
- Reduce rate of alloimmunization to leukocyte antigens for patients with hematologic malignant disease
- Reduce risk of transmission of cytomegalovirus among persons at risk of infection by transfusion

**Emerging Indications**

- Improved outcomes in cardiac surgery

**Indications under Investigation\***

- Reduce risk of bleeding due to platelet refractoriness
- Reduce risk of postoperative bacterial infection
- Reduce risk of immune modulation leading to tumor recurrence
- Reduce risk of bacterial overgrowth of *Yersinia enterocolitica* in stored RBCs
- Reduced risk of white-cell-associated viruses (Epstein-Barr virus, human T-cell lymphotropic virus)
- Reduce, but not eliminate risk of transfusion-associated graft-vs-host disease (GVHD)

**Contraindications†**

- Prevention of GVHD
- Prevention of transfusion-related acute lung injury caused by passive infusion of leukocyte antibody
- Prevention of the red cell or platelet storage lesion
- Prevention of virus reactivation among patients with human immunodeficiency virus infection
- Prevention of vCJD transmission

\* Inadequate evidence to suggest benefit from leukocyte reduction.

† Evidence demonstrates no benefit of leukocyte reduction.

per transfusion. This value represents 100-fold more leukocytes than a widely used benchmark for prevention of HLA alloimmunization or CMV transmission— $<5 \times 10^6$  per transfusion.

**Mechanisms**

At least three mechanisms may account for the development of FNHTRs (Fig 16-6) (see Chapter 53). The final pathway for all three mechanisms is the elaboration of inflammatory cytokines such as IL-1, IL-6, or TNF. IL-1 and TNF react with cell surface receptors in the hypothalamus and stimulate prostaglandin and leukotriene-mediated pathways that reset the thermoregulatory center of the brain. IL-6 stimulates energy mobilization in muscle and fatty tissue, which leads to increased body temperature.

One mechanism of FNHTRs involves recipient antibodies reacting with donor leukocytes and stimulating the release of endogenous pyrogens (cytokines) from the donor cells. This mechanism is consistent with the prevention of FNHTRs by leukocyte reduction of donor blood and with the results of studies by Perkins et al<sup>31</sup> and Brubaker<sup>32</sup> that documented the high prevalence of lymphocyte or granulocyte antibodies in the sera of patients who experienced FNHTRs. The mechanism does not explain all FNHTRs,

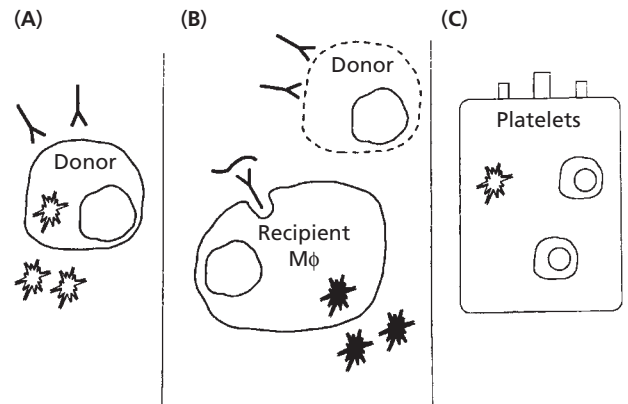


**Figure 16-6.** Three mechanisms for the development of febrile nonhemolytic transfusion reactions. (A) Donor cells react with recipient leukocyte antibody and cause a release of interleukin of donor origin. (B) Donor cells react with recipient antibody and form antigen-antibody complexes that react with recipient monocytes to result in the release of recipient interleukin. (C) Residual donor leukocytes present in platelet concentrates during storage release interleukin passively transfused to the recipient.

however. Patients with antibodies to platelet-specific antigens may have febrile reactions when they receive LR platelet concentrates.

A second proposed mechanism for FNHTRs suggests that inflammatory cytokines are released by recipient cells in response to antigen-antibody complexes formed when incompatible donor cells are transfused to patients with reacting antibodies.<sup>33</sup> This mechanism is consistent with the role of recipient antibodies, the ease of preventing FNHTRs to RBCs, and the difficulty of preventing these reactions among alloimmunized patients receiving platelet concentrates. This mechanism also accounts for the fever and chills accompanying hemolytic transfusion reactions.

A third mechanism of FNHTRs is based on the passive transfer of inflammatory cytokines that accumulate in whole-blood-derived platelet concentrates during storage. Several investigators have found detectable levels of IL-1, TNF, and IL-6 in platelet concentrates after several days of storage.<sup>34</sup> These substances do not accumulate in platelet concentrates that have undergone prestorage leukocyte reduction or in platelet concentrates prepared from buffy coats. Of note, the accumulation of IL-1 and TNF in platelet concentrates does not occur if platelet concentrates are stored at refrigerated temperatures.<sup>35</sup> This may explain why IL-1 does not accumulate in RBC units and may account for the relative ease of preventing FNHTRs to RBCs.

Clinical evidence that FNHTRs to platelet concentrates result from passive transfer of cytokines was presented by Heddle et al.<sup>36</sup> They showed that the large majority of FNHTRs were to the plasma supernatant from stored whole-blood-derived platelets vs the cellular (platelet fraction). Furthermore, the severity of the reactions to the plasma supernatant correlated with the level of IL-1 $\beta$  and IL-6 present in the plasma. The passive transfer of cytokines accumulated during storage of platelet concentrates may explain FNHTRs that occur in the absence of detectable

**Table 16-5.** Effect of Prestorage Leukocyte Reduction on the Rate of FNHTRs

| Author                | Non-LR<br>RBCs | LR<br>RBCs | Non-LR<br>Plts | LR<br>Plts |
|-----------------------|----------------|------------|----------------|------------|
| Yazer <sup>39</sup>   | 0.33%          | 0.19%      | 0.45%          | 0.11%      |
|                       |                | p < 0.001  |                | p < 0.001  |
| Paglino <sup>40</sup> | 0.34%          | 0.18%      | 2.18%          | 0.15%      |
|                       |                | p < 0.0001 |                | p < 0.0001 |
| King <sup>41</sup>    | 0.37%          | 0.19%      | NA             | NA         |
|                       |                | p = 0.0008 |                |            |

Percentages refer to recipients with FNHTRs.  
FNHTRs = febrile nonhemolytic transfusion reactions; LR = leukocyte-reduced;  
RBCs = Red Blood Cells; Plts = platelets.

leukocyte or platelet antibodies or the absence of a history of transfusions or pregnancies. It is also consistent with the observation that the incidence of FNHTRs to platelet concentrates increases with increasing duration of storage.<sup>37,38</sup>

Clinical Studies

Several recent clinical trials have confirmed that prestorage leukocyte reduction is effective in reducing the rate of FNHTRs to red cells by approximately 50% with residual rates well below 1% (Table 16-5).<sup>39-41</sup> In contrast to the experience with RBCs, leukocyte reduction has had mixed results regarding the prevention of FNHTRs to platelet concentrates. Consistent with the role of passively transferred cytokines as mediators of reactions to platelet concentrates, Mangano et al,<sup>42</sup> Goodnough et al,<sup>43</sup> and Mintz<sup>44</sup> found that bedside leukocyte reduction of platelet concentrates did not substantially reduce the rate of FNHTRs. In contrast, prestorage leukocyte reduction of platelet concentrates has resulted in a major reduction in the rate of FNHTRs to less than 1%.<sup>39,40</sup>

Reduction in HLA Alloimmunization

Leukocyte reduction is commonly used in an attempt to prevent alloimmunization to donor HLA antigens. Prevention of alloimmunization is of clear clinical value for patients undergoing renal, heart, or lung transplantation<sup>45</sup> and those with aplastic anemia awaiting stem cell transplantation.<sup>46</sup> In addition, much attention has been given to the prevention of alloimmunization among patients undergoing chemotherapy. In 33 studies involving more than 3000 patients, the median reported incidence of HLA alloimmunization when unmodified components were used was 39% (range, 20% to 71%). The use of LR components was shown to reduce the incidence of HLA alloimmunization and subsequent platelet refractoriness. Reduced refractoriness is expected to decrease morbidity or mortality caused by bleeding, but this has not yet been documented in any clinical trials.

The threshold level of leukocytes needed to provoke primary HLA alloimmunization is generally regarded to be 1 to 5 × 10<sup>6</sup> per transfusion. Fisher et al<sup>47</sup> administered transfusions of small doses of platelet concentrate to two groups of 12 patients

undergoing dialysis on three occasions at 14-day intervals. Five of 12 patients given a mean of 15 × 10<sup>6</sup> WBCs per transfusion made HLA antibodies, and none of 12 given fewer than 5 × 10<sup>6</sup> WBCs per transfusion made HLA antibodies. Although this study had numerous serious design flaws, results of a subsequent study by van Marwijk-Kooy et al<sup>48</sup> also suggested that 5 × 10<sup>6</sup> represented a threshold dose for alloimmunization. In a prospective trial, patients with leukemia received filtered buffy-coat-depleted RBCs and were then randomly assigned to receive either centrifuged platelet concentrates (mean WBC content, 35 × 10<sup>6</sup>) or filtered platelet concentrates (mean WBC content 5 × 10<sup>6</sup>). Clinical refractoriness occurred in 46% of those given centrifuged platelet concentrates but in only 11% of those given filtered platelet concentrates. Although significant individual variation is likely, 5 × 10<sup>6</sup> WBCs per transfusion was adopted during the 1990s as the standard for LR blood components. However, the threshold level was set at 1 × 10<sup>6</sup> WBCs per transfusion in Europe.

Mechanism

Decades of research provide unequivocal evidence that passenger leukocytes, which express donor HLA antigens, are a prime cause of recipient sensitization to HLA alloantigens. Experiments by Claas et al<sup>49</sup> with a rodent transfusion model showed that residual donor leukocytes provoked major histocompatibility complex (MHC) alloimmunization and that leukocyte reduction could decrease the incidence of primary sensitization to MHC antigens. These investigators further documented that even very low numbers of donor leukocytes in previously sensitized animals induced secondary immunization, suggesting that leukocyte reduction would be less effective in the previously sensitized patient. The importance of donor leukocytes was confirmed in experiments by Kao<sup>50</sup> and Blajchman et al,<sup>26</sup> who also provided evidence for the possible role of antigens on microparticles in plasma.<sup>51</sup>

Alloimmunization can occur by means of both direct and indirect immune recognition.<sup>52</sup> Direct allorecognition is the process by which recipient immune cells respond directly to donor HLA antigens without the processing of donor antigens by recipient antigen-presenting cells (APCs). The mixed lymphocyte reaction is an in-vitro example of direct T-cell recognition. Direct allosensitization requires at least three fundamental elements—binding of the antigen to the antigen receptor, binding of costimulatory molecules mediating cell-cell contact, and local elaboration of cytokines and appropriate cytokine receptors. Recipients may recognize intact Class I structures on the surface of donor leukocytes (Fig 16-7). Alternatively, Class-II-positive donor APCs may carry, within the peptide-binding groove, oligopeptides that represent the cell's own HLA Class I antigen (Fig 16-8). In either case, depletion of Class-II-positive donor APCs would reduce the chance of direct HLA immunization to Class I antigens. The failure of the recipient to recognize donor Class I antigens on platelets may reflect the fact that platelets lack critical costimulatory molecules required for direct allostimulation.

Indirect allorecognition is the process by which recipient APCs first engulf donor cells and then process donor antigen

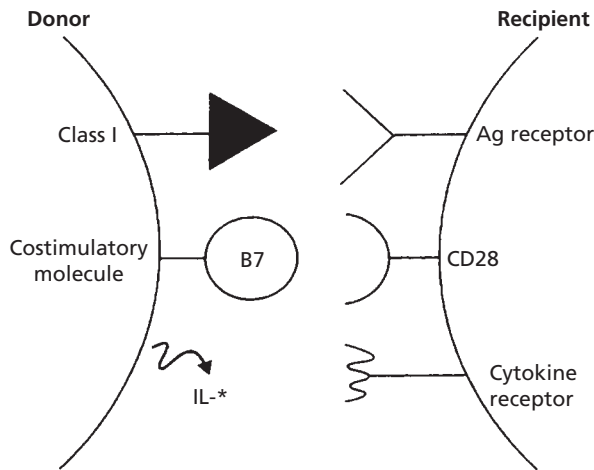


**Figure 16-7.** Direct HLA alloimmunization to Class I antigen. The recipient cell directly recognizes foreign Class I provided that a second costimulatory molecule also is expressed and provided that the appropriate cytokine is locally present. Ag = antigen; IL = interleukin.

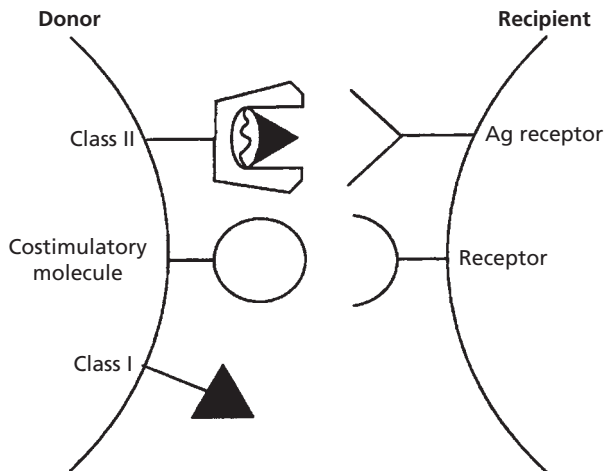


**Figure 16-8.** Direct HLA alloimmunization to Class I antigen. The donor cell carries a fragment of its Class I molecule within the peptide-binding groove of its own Class II molecule. This model, suggested by Dzik, provides one hypothesis to explain the frequency with which transfusion of unmodified cellular components results in antibodies to HLA Class I. Ag = antigen.

for redisplay to the recipient immune system. Donor cells, cell fragments, or soluble donor antigens are engulfed and degraded within lysozymes of the recipient APCs. Small peptide fragments corresponding to the alloantigenic region of donor HLA are then deposited in the peptide-binding groove of Class II structures on recipient APCs (Fig 16-9).<sup>53</sup> Recipient T and B cells then interact with recipient APCs in an MHC-restricted manner and respond to the donor peptide antigens displayed by the APCs. Recipient helper T-cell receptor-mediated recognition of this complex results in elaboration of cytokines by the donor APC (IL-1, IL-12), which in turn results in APC surface expression of CD80 and CD86. These molecules bind to CD28, the

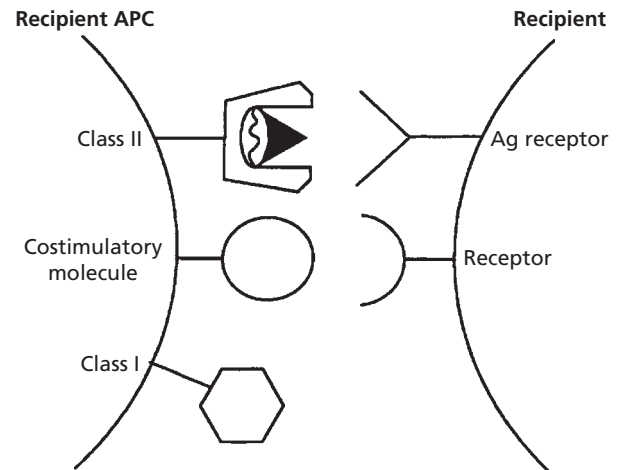


**Figure 16-9.** Indirect HLA alloimmunization to Class I antigen. Recipient antigen-presenting cells (APCs) have digested donor cells or cell fragments and deposited a fragment of donor Class I antigen in the peptide-binding groove of the recipient Class II molecule.

central co-stimulatory molecule on helper T cells. T cells are further activated and elaborate cytokines such as CD40 ligand, IL-4, IL-5, and IL-10, which provide the critical help to B lymphocytes for their proliferation and antibody production.<sup>52</sup> Ultraviolet (UV) B irradiation of APCs disrupts costimulatory signals<sup>54</sup> and thus may account for the effectiveness of this method for reducing the rate of HLA alloimmunization observed in a large clinical trial.<sup>55</sup> Indirect immune recognition is unlikely to account for the initial response in an in-vitro mixed leukocyte reaction. However, the indirect pathway may be particularly relevant for alloimmune responses observed in vivo after transfusion.<sup>52</sup> In addition, the indirect pathway may account for experimental evidence in favor of alloimmunization by donor cell microparticles.

### Clinical Trials

Several clinical trials have investigated the usefulness of leukocyte reduction for the prevention of HLA alloimmunization and refractoriness. These studies have been reviewed by Heddle<sup>56</sup> and by Vamvakas.<sup>57</sup> Twelve randomized, controlled trials (RCTs) have been published (Table 16-6). Typically, in these trials patients with hematologic malignant disease were randomly assigned to receive either filtered or unfiltered blood components. Filters have ranged from early devices that achieved only 1- to 1.5-log reduction in residual leukocytes to current filters that are capable of at least 3-log reduction. Some investigators measured the residual WBC content after filtration, whereas others assumed that patients in the filtration arm received components that had been adequately leukocyte reduced. Some investigators used in-laboratory leukocyte reduction, whereas others used bedside leukocyte filtration. Patients were followed with periodic measurement of HLA antibodies. The details of HLA antibody detection and the criteria for labeling a patient as alloimmunized varied among different clinical trials. Once a patient met

**Table 16-6.** Prospective, Controlled Clinical Trials of Leukocyte Reduction to Decrease HLA Alloimmunization\*

| Author       | Diagnosis       | Method of Leukocyte Reduction |            | All Patients  |               | Previously Immunized |              |
|--------------|-----------------|-------------------------------|------------|---------------|---------------|----------------------|--------------|
|              |                 | RBCs                          | PLTs       | Control       | LR            | Control              | LR           |
| Elghouzzi    | Solid tumor     | Cellselect                    | None       | 26/93 (0.28)  | 10/67 (0.15)  | 27/73 (0.32)         | 10/52 (0.19) |
| Schiffer     | AML             | Deglyced                      | Pooled     | 13/31 (0.42)  | 5/25 (0.20)   | 9/19 (0.47)          | 1/10 (0.10)  |
| Andreu       | Heme malignancy | IG 500                        | IG 500     | 11/35 (0.31)  | 4/34 (0.12)   | 4/14 (0.28)          | 2/13 (0.15)  |
| Sniecinski   | AML, ALL, AA    | IG 500                        | IG 500     | 10/20 (0.50)  | 3/20 (0.15)   | 6/12 (0.50)          | 2/10 (0.20)  |
| Rebulla      | AML, ALL        | Cellselect                    | IG 500     | 5/15 (0.33)   | 4/16 (0.25)   | 2/5 (0.40)           | 2/4 (0.50)   |
| van Marwijk  | AML, ALL        | Cellselect                    | Cellselect | 11/26 (0.42)  | 2/27 (0.07)   | 2/6 (0.33)           | 1/5 (0.20)   |
| Oksanen      | AML, ALL BMT    | Cellselect                    | IG 500     | 3/15 (0.20)   | 2/16 (0.12)   | 1/3 (0.33)           | 1/4 (0.25)   |
| Handa        | Heme malignancy | R500                          | PL10N      | 9/23 (0.39)   | 4/49 (0.08)   | 4/6 (0.66)           | 1/20 (0.05)  |
| Lane         | AML, CLL, CML   | Filtration                    | Filtration | 7/20 (0.35)   | 3/26 (0.11)   | 4/10 (0.40)          | 3/9 (0.33)   |
| Williamson   | Heme malignancy | Filtration                    | Filtration | 21/56 (0.37)  | 15/67 (0.22)  | —                    | —            |
| Sintnicolaas | AML             | Filtration                    | Filtration | —             | —             | 9/21 (0.43)          | 11/25 (0.44) |
| TRAP         | AML             | Filtration                    | Filtration | 59/131 (0.45) | 25/137 (0.18) | 30/48 (0.62)         | 17/53 (0.33) |

\*Data derived from Heddle<sup>56</sup> and Vamvakas.<sup>57</sup>

†Numbers are the proportion (%) of patients demonstrating HLA antibodies.

LR = leukocyte reduced; RBCs = Red Blood Cells; PLTs = platelets; AML = acute myelogenous leukemia; ALL = acute lymphocytic leukemia; AA = aplastic anemia; BMT = bone marrow transplantation; CLL = chronic lymphocytic leukemia; CML = chronic myelogenous leukemia.

the study criteria for alloimmunization, some trials censored the patient from further analysis in the study, whereas others continued to collect data on the patient. Because of these differences in study design and execution, strict comparison of the current RCTs must be made cautiously. With these limitations, meta-analysis has identified a reduction in alloimmunization with leukocyte reduction for patients with hematologic malignant disease.<sup>56,57</sup> In support of these studies, a decrease in platelet alloimmunization and refractoriness was observed in a Canadian study of nearly 14,000 platelet transfusions with the adoption of universal leukocyte reduction.<sup>58</sup> Of particular note, RCTs of leukocyte reduction for the prevention of HLA alloimmunization among less immunosuppressed transfusion recipients are lacking. A retrospective study of leukocyte reduction for potential kidney transplant recipients found no effect on HLA alloimmunization rates among those who received LR (33%) vs non-LR (27%) blood components.<sup>59</sup>

The Trial to Reduce Alloimmunization to Platelets (TRAP) was the largest randomized trial to test the effect of leukocyte reduction for prevention of HLA alloimmunization.<sup>55</sup> The 530 patients undergoing initial therapy for acute leukemia were randomly assigned to one of four arms—whole-blood-derived platelets (control), LR whole-blood-derived platelets, LR apheresis platelets, and UVB-irradiated pooled platelets. All red cell components were leukocyte reduced. Patients were evaluated for 8 weeks. Blood components were leukocyte reduced in the laboratory under process control. Overall, 3% of transfusions were not of the assigned group. Alloimmunization was defined as cytotoxicity against one or more wells of a 30- to 60-cell target HLA antibody panel. During the 2 weeks before enrollment and assignment, 75% of patients had received transfusions (mostly

non-leukocyte-reduced). The results showed that platelet refractoriness was infrequent (10%) and was similar for all four treatment arms. Lymphocytotoxic antibodies developed in 45% of patients supported with non-LR pooled platelets compared with 21% in the UVB group, 18% in the group receiving LR pooled platelets, and 17% in the group receiving LR apheresis platelets. All three treatment groups had significantly lower rates of alloimmunization than did controls. The three treatment groups had similar results; that is, no advantage was observed for prestorage LR apheresis platelets compared with poststorage LR pooled platelets. It is also noteworthy that alloimmunization was defined as reactivity against a single target cell and so should not be confused with broad alloimmunization. Finally, the study was limited to 8 weeks; long-term rates of alloimmunization were not determined.

### Will Leukocyte Reduction Prevent Secondary Alloimmunization?

Because a secondary or anamnestic immune response requires a far smaller antigenic challenge than does a primary response, the secondary antibody recall to MHC antigens is difficult to prevent with LR blood components.<sup>49</sup> Most clinical studies of HLA alloimmunization have included patients with previous exposure to transfusion or pregnancy. Therefore, the issue of secondary alloimmunization seldom has been independently addressed. However, subgroup analysis of studies listed in Table 16-6 showed a higher rate of sensitization among pregnant patients or those who previously have received transfusions. The issue of secondary alloimmunization was directly addressed in the study by Sintnicolaas et al.<sup>60</sup> Female patients (n = 75) with hematologic malignant disease and a history of previous pregnancy were

randomly assigned to receive platelet concentrate support with either unmodified or LR apheresis platelet concentrates. Among the evaluable patients, HLA alloimmunization developed in 43% (9 of 21) of the standard platelet concentrate group and in 44% (11 of 25) of the filtration group. Refractoriness occurred in 41% (14 of 34) of women in the standard platelet concentrate group and in 29% (8 of 28) of the filtration group ( $p=0.52$ ). The time to development of refractoriness was similar in the two groups. The authors concluded that leukocyte reduction to fewer than  $5 \times 10^6$  WBCs per transfusion did not prevent alloimmunization and refractoriness among previously sensitized recipients.

In the TRAP study, however, analysis of patients who had been pregnant showed some benefit, albeit decreased, from the use of LR blood. In that study, 62% of patients with a history of pregnancy had HLA alloantibodies after transfusion of routine components; 33% did so after transfusion of LR components.<sup>55</sup> More recently, Seftel et al analyzed 106 women with hematologic malignancy and a history of pregnancy treated before and after the introduction of universal leukocyte reduction in Canada.<sup>58</sup> They found that leukocyte reduction did not significantly change the rate of alloimmunization (15% vs 10%) or platelet refractoriness (11% vs 9%), although the surprisingly low rate of alloimmunization in control patients may have accounted for the lack of observed benefit of leukocyte reduction. However, when previous transfusion was studied as an immunizing event, leukocyte reduction was highly effective in reducing the rate of alloimmunization (29% vs 2%).<sup>58</sup> Thus, it appears that patients with hematologic malignant disease and a history of exposure to foreign HLA antigens from a previous pregnancy derive diminished benefit from the use of LR components. This benefit may not extend to patients with diagnoses other than hematologic malignant disease.

#### **Will Prevention of Alloimmunization Decrease Bleeding Complications Caused by Platelet Refractoriness?**

Although the clinical trials conducted to date provide meaningful information on the prevention of HLA alloimmunization by means of leukocyte reduction, measurement of alloimmunization is a poor proxy for prevention of bleeding caused by immune-mediated refractoriness and is no proxy for bleeding resulting from nonimmune causes.<sup>56</sup> In addition, because bleeding is not limited to refractory patients, prevention of refractoriness will not prevent all bleeding complications. Finally, because serious bleeding complications are infrequent even when unmodified (non-LR) blood components are used, the potential gain resulting from the prevention of alloimmunization is small. These three factors make the clinical importance of leukocyte filtration technology difficult to assess. For example, in one prospective study, 502 patients undergoing marrow transplantation were randomly assigned to receive support with either filtered or unfiltered blood components to analyze the effect of filtration on transmission of CMV.<sup>12</sup> Even among patients who were at high risk of fatal bleeding complications, there was no difference in overall early mortality between the two treatment arms. Thus, leukocyte filtration was not likely to have substantially

affected the rate of fatal bleeding episodes. Similarly, the large TRAP study was unable to demonstrate that the use of LR blood conferred any advantage to prevent bleeding due to HLA refractoriness. Moreover, survival rates, remission rates, and blood component support all were unaffected by leukocyte reduction. Because of the low incidence of bleeding associated with platelet refractoriness, prevention of HLA alloimmunization by means of leukocyte reduction may not be cost-effective. Thus, leukocyte reduction has been shown to decrease the rate of HLA alloimmunization, but the effect on patient outcome has not been adequately assessed.

#### **Reduced Risk of Transmission of Cytomegalovirus and Other Leukotropic Agents**

The human leukotropic viruses—CMV, Epstein-Barr virus (EBV), and human T-cell lymphotropic virus (HTLV-I/II)—reside within the leukocytes of infected individuals. These viruses are found in such a low copy number outside of cells, that plasma from donors with established infections does not transmit infection. Unlike human immunodeficiency virus (HIV), these three viruses are not present in platelets. Thus, substantial leukocyte reduction of blood components would be expected to prevent transmission of CMV, EBV, and HTLV through blood transfusion. Leukocyte reduction does appear to reduce the content of EBV<sup>61</sup> and HTLV<sup>62</sup> in cellular components; however, EBV and HTLV are rarely clinically important transfusion-transmitted pathogens. In contrast, CMV is an important pathogen for immunosuppressed recipients and has been the subject of several clinical trials of LR blood components<sup>12,63</sup> (Table 16-7). In evaluating such trials, it is important to recognize that many patients, even if immunosuppressed, do not acquire primary CMV infection from unmodified blood transfusions. This may be attributed to the fact that among healthy blood donors only a small minority of leukocytes (estimated at less than 0.2%) carry CMV.<sup>64</sup> Sensitive PCR techniques are needed to demonstrate the CMV genome in the blood of seropositive healthy donors. Studies on blood from infected donors showed that the CMV PCR signal is lost or greatly reduced when blood was leukocyte reduced by means of filtration<sup>65-67</sup> or apheresis processing.<sup>66</sup>

Collection of blood from donors with negative test results for antibodies to CMV has been a traditional method to decrease the risk of transfusion-transmitted CMV. Although the use of CMV-seronegative blood has been shown to be an effective prevention strategy, it is not completely effective. Using an older PCR assay, Larsson et al<sup>68</sup> reported that CMV DNA could be detected in 55% of seronegative Swedish donors. A more recent study using a CMV nucleic acid amplification test with superior specificity found no positives among 514 seronegative donors, suggesting that the results reported in the Larsson study were false-positive results.<sup>64</sup> However, among high-risk recipients even the use of seronegative components is associated with a 1% to 1.5% failure rate. This may be caused by the presence of low level CMV viremia in recently seroconverting donors reported to be 1.6%.<sup>69</sup> Because the use of CMV-seronegative donors or

**Table 16-7.** Clinical Trials of Cytomegalovirus Prevention by Leukocyte Reduction\*

| Author        | Design                 | Diagnosis     | Method of Leukocyte Reduction |                  | No. of CMV Seroconversions <sup>†</sup> |           |
|---------------|------------------------|---------------|-------------------------------|------------------|-----------------------------------------|-----------|
|               |                        |               | RBCs                          | PLTs             | Unmodified                              | LR        |
| Murphy        | Retrospective          | Leukemia      | Bedside filter                | Centrifuge       | 2/9 (22)                                | 0/11 (0)  |
| Graan-Hentzen | Retrospective          | Mixed         | Bedside filter                | Centrifuge       | 10/86 (12)                              | 0/59 (0)  |
| Eisenfield    | Retrospective          | Newborn       | Bedside filter                | —                | —                                       | 0/48 (0)  |
| Ohto          | Prospective randomized | Newborn       | Filter                        | —                | 1/19(5)                                 | 3/33(9)   |
| Gilbert       | Prospective randomized | Newborn       | Bedside filter                | —                | 9/42 (21)                               | 0/30 (0)  |
| Verdonck      | Prospective            | Auto/allo BMT | Filter                        | —                | —                                       | 0/29 (0)  |
| De Witte      | Prospective            | Allo BMT      | Filter                        | Centrifuge       | —                                       | 0/28 (0)  |
| Bowden        | Prospective randomized | Auto/allo BMT | Bedside filter                | Centrifuge       | 7/30 (23)                               | 0/35 (0)  |
| Bowden        | Prospective randomized | Auto/allo BMT | Bedside filter                | Bedside filter   | 3/246 (1)                               | 5/241 (2) |
| Pamphilon     | Observational          | Allo BMT      | NR                            | NR               | 0/114                                   | 0/62      |
| Nichols       | Observational          | Allo BMT      | Filter                        | Filter/apheresis | 6/360(2)                                | 18/447(4) |

\*Data derived from Bowden<sup>12</sup> and Hillyer.<sup>63</sup>

<sup>†</sup>Values in parentheses are percentages.

LR = leukocyte reduced; RBCs = Red Blood Cells; PLTs = platelets; CMV = cytomegalovirus; BMT = bone marrow transplantation.

leukocyte reduction does not completely prevent CMV transmission, products prepared with either of these methods are better termed “reduced risk” for CMV.

### Transfusion-Transmitted Cytomegalovirus among Low-Birthweight Neonates

Term neonates of normal birthweight with normal immunity do not acquire serious CMV disease as a result of exposure to blood components. However, low-birthweight premature neonates, who frequently need small-volume blood transfusions, are at risk of contracting CMV disease (see Chapter 47). Consequently, all low-birthweight infants are appropriate candidates for strategies to prevent CMV transmission by means of transfusion. Because these infants are at times given fresh blood, protection against CMV resulting from leukocyte apoptosis during refrigerated storage is diminished. Early studies showed that the use of frozen deglycerolized RBCs decreased the rate of transmission of CMV among at-risk neonates. As high-performance leukocyte reduction filters replaced freezing technology, studies were performed to document the effectiveness of leukocyte depletion to prevent CMV transmission to at-risk neonates. Gilbert et al<sup>70</sup> conducted a prospective, randomized, blinded trial in which 515 newborns were randomly assigned to receive transfusion support with either unmodified RBCs or RBCs filtered through an IG500 (Terumo Corporation) filter capable of 1- to 1.5-log leukocyte removal. In the control arm, 29 infants weighed less than 1500 g, and CMV infection developed in nine (33%). In the filtration arm, among 24 neonates weighing less than 1500 g who were CMV seronegative and received CMV-seropositive filtered blood, none acquired CMV infection. In another study, Eisenfield et al<sup>71</sup> used less advanced filtration technology and documented prevention of CMV transmission to at-risk neonates. In contrast,

Ohto et al<sup>72</sup> performed a prospective randomized study of LR blood components in 52 newborn infants of whom 43 weighed <1500 g. There was no effect of leukocyte reduction on the rate of CMV infection; however, the geographic region in Japan where this study was conducted had a high rate of CMV among the mothers (89%) and the blood donors (89%). Because current filters are considerably more effective at leukocyte reduction than were those used in the published studies, it is likely that filtration technology is completely adequate to prevent CMV transmission to at-risk neonates. There are no data to support additional benefit by using a combination of CMV-seronegative blood donors and leukocyte reduction in low-birthweight neonates. The presence of a passively transferred antibody must be considered in interpretation of the results of CMV serologic testing after transfusion.

### Transfusion-Transmitted Cytomegalovirus after Hematopoietic Transplantation

Patients undergoing allogeneic hematopoietic transplantation are at great risk of CMV infection and disease because of the intense immunosuppression needed for stem cell engraftment, graft-vs-host disease (GVHD), and the persistent defects in cellular and humoral immunity resulting from the chimeric immune system present after transplantation. In this setting, uncontrolled CMV infection can represent a devastating illness with high mortality. As a result, CMV-seronegative recipients of CMV-seronegative stem cells should be protected from transfusion-transmitted CMV. When either the donor or the recipient is seropositive for CMV, the role of transfusion-transmitted CMV becomes much less important. In these latter cases, there is little evidence that the use of CMV-reduced-risk blood components has any substantial clinical benefit.

For CMV-seronegative donor-recipient pairs, the traditional approach has been to use seronegative donor blood. Early reports documented that leukocyte filtration was an alternative approach to prevent transfusion-transmitted CMV infection among patients undergoing allogeneic marrow transplantation. The results of these studies were consistent with those of studies conducted with neonates. They showed that prevention of CMV transmission with use of platelet concentrates does not require extensive leukocyte reduction. Bowden et al<sup>12</sup> conducted a large, randomized, prospective trial with CMV-negative patients who received transplants of CMV-negative marrow. The investigators compared support with CMV-seronegative blood with support with filtered blood from untested donors. They randomly assigned 502 CMV-seronegative patients undergoing autologous or allogeneic marrow transplantation to two treatment groups. Among 252 patients receiving CMV-seronegative blood components, two had CMV infection; none had CMV disease. Among 250 patients receiving filtered CMV-untreated components, three CMV infections (three with CMV disease) were observed. Two additional CMV infections in the seronegative group and three in the filtration group occurred within 21 days of entrance to the study and were considered the result of transfusions before enrollment. The survival rate was equal in the two study arms.

Several features of the study by Bowden et al<sup>12</sup> affect interpretation of the results. First, the filters used were not as effective as current filters. Second, filtration was conducted at the bedside under conditions now known to decrease filter performance. Third, patients could receive up to 6 units of blood to which they were not assigned (protocol violations) and still have the outcome included in the results. Fourth, the serostatus of four of the five patients who developed CMV infection within 3 weeks of being assigned to treatment groups was equivocal or discrepant. Although most observers interpreted this study to show no significant difference between the two strategies of CMV prevention, the results sparked controversy over the relative merits of the two methods. A subsequent nonrandomized “before and after” trial by Pamphilon et al<sup>73</sup> compared 114 recipients of CMV-seronegative components to 61 recipients of LR components. There were no cases of CMV infection observed in either group. However, a larger “before and after” study of 807 patients prospectively followed by Nichols et al<sup>74</sup> reported that CMV-infection rose from 1.7% when almost exclusively CMV-seronegative components were used to 4.0% when filtered components accounted for 1.5% of red cells and 15.8% of platelets transfused. Only a single case of CMV disease was observed because of CMV antigen monitoring and preemptive antiviral therapy. This study was not randomized and compared patients transfused in 1994 through 1996 to those transfused in 1996 through 2000. Observed differences may have been caused by confounders such as changes in treatment regimens or leukocyte reduction methods. In addition, the source of CMV was not identified and patients who received more transfusions were more likely to develop CMV infection and also more likely to receive a filtered component. There are no studies comparing the use of both methods of CMV prophylaxis

vs either method alone because of the large sample size required. The controversy regarding the equivalence of CMV-seronegative vs LR components was recently reviewed and a meta-analysis performed on the clinical studies in allogeneic stem cell recipients.<sup>75</sup> The meta-analysis indicates that CMV-seronegative components are associated with a 60% risk reduction for CMV infection compared with leukocyte reduction. This conclusion should be qualified in that the findings may have been biased by confounders in the observational studies by Nichols<sup>74</sup> and Pamphilon<sup>73</sup> and are applicable only to allogeneic stem cell recipients. With active CMV surveillance and preemptive antiviral therapy in this population, there is no evidence that clinical outcomes are worse with LR components.

### **Transfusion-Transmitted Cytomegalovirus among Recipients of Solid-Organ Transplants**

Although the morbidity and mortality associated with CMV among recipients of solid-organ transplants are not as severe as they are among recipients of allogeneic marrow, CMV still represents a serious infectious complication of solid-organ transplantation. In addition, CMV infection can promote rejection of the allograft. Because the graft is the most common site of post-transplantation CMV infection, the distinction between rejection and CMV infection can be difficult. Glomerulopathy, vanishing bile duct syndrome, accelerated atherosclerosis, and obliterative bronchiolitis may be the renal, hepatic, cardiac, and lung allograft expressions of CMV infection. The dominant factors controlling the development of CMV infection after transplantation are the serologic status of the organ donor and recipient and the degree of immunosuppression of the recipient. There is no evidence that patients who are CMV seropositive benefit from use of seronegative or LR blood components. Although infection with a second strain of CMV transmitted by blood transfusion is theoretically possible, there are no published reports of second-strain infections attributable to blood transfusion. Patients who are CMV seronegative and who receive organs from CMV-seropositive donors are at the greatest risk of postoperative CMV disease and acquire the infection from the allograft. Thus, prevention of transfusion-transmitted CMV is currently an appropriate consideration only for CMV-seronegative recipients of allografts from CMV-seronegative donors.<sup>45</sup>

No results of prospective, randomized trials document that leukocyte reduction prevents transfusion-transmitted CMV infection among seronegative recipients of seronegative solid organs. Experience with neonates, patients with hematologic malignant disease, and patients undergoing hematopoietic transplantation, however, suggests it is highly likely that use of CMV-seronegative or LR components are equivalent methods of reducing the risk of transfusion-transmitted CMV infection. Liver transplantation often requires massive transfusion support, but the attack rate for transfusion-transmitted CMV in liver transplantation is very low. In many cases it is not practical to restrict these patients to seronegative or LR blood components. For example, the deliberate use of CMV-untreated, non-LR



blood in the care of CMV-seronegative patients receiving CMV-seronegative liver allografts resulted in only a 10% incidence of CMV disease and no deaths attributed to CMV infection.

## Investigative Applications of Leukocyte Reduction

### Virus Reactivation

A number of preclinical studies suggested a role of donor leukocytes in CMV and HIV reactivation.<sup>76,77</sup> The clinical relevance of these observations was specifically addressed in a large, multicenter, RCT—the Viral Activation by Transfusion Study (VATS).<sup>78</sup> The study was conducted to evaluate the ability of LR blood to prevent either HIV or CMV activation among a cohort of patients with HIV infection undergoing therapy for the infection. The study showed no beneficial effect of leukocyte reduction for prevention of early death, virus activation, or time to first infection-related complication.

### Bacterial Overgrowth

It is unlikely that prestorage removal of leukocytes would have any measurable effect on the incidence of bacterial overgrowth in blood components. The mechanisms by which leukocyte reduction filters may deplete blood components of low levels of contaminating bacteria have been reviewed.<sup>79</sup> Because bacterial overgrowth is infrequent, a clinical study would require an enormous number of observations. The hemovigilance network in France reported an analysis of reported cases of bacterial sepsis before and after introduction of universal leukocyte reduction.<sup>80</sup> The study found that bacterial sepsis decreased from 3.8% to 1.7% ( $p < 0.001$ ) of all reported adverse events.<sup>80</sup> This observation does not prove that leukocyte reduction accounts for the reduced reports of bacterial sepsis. In the absence of a clinical trial, investigation of the potential role of prestorage filtration has entailed deliberate inoculation experiments. Units of blood are “spiked” with varying concentrations of bacteria and split into pairs, one of which is filtered. The paired units are stored and checked periodically for bacterial overgrowth. Many variables affect the results of such experiments. These include the strain of bacteria, the presence of bacterial plasmids, blood component characteristics, the filter selected, the inoculation dose, the holding time and temperature between inoculation and filtration, the method of assaying for bacterial overgrowth, the type of isolator used to culture bacteria, and the statistical power of the study.<sup>79</sup> Because of these numerous variables, caution must be used when interpreting the results of in-vitro experiments. It is expected that pathogen reduction systems will prove far more effective than leukocyte reduction for reducing the risk of bacterial overgrowth in stored blood components.

### Variant Creutzfeldt-Jakob Disease

Universal leukocyte reduction was adopted by several European countries to reduce the risk of variant Creutzfeldt-Jakob disease (vCJD) transmission based on experimental evidence of B-cell depletion in an animal model.<sup>81</sup> A subsequent study has shown

that prions can be found in both plasma and cellular elements of blood and that leukocyte reduction with current filters reduces infectivity by approximately 40%, but does not eliminate infectivity.<sup>82</sup> Novel blood filters currently under development are specifically designed for the removal of prion protein.<sup>83</sup> However, currently no claim can be made that leukocyte reduction has a clinical impact on transfusion-transmitted vCJD.

### Transfusion-Related Acute Lung Injury

Transfusion-related acute lung injury (TRALI) is an acute, immune-mediated transfusion reaction characterized by dyspnea, hypoxia, pulmonary edema, low pulmonary capillary wedge pressure, and alveolar infiltrates on chest radiographs (see Chapter 56). Two different mechanisms may contribute to the development of TRALI. First, results of serologic investigations suggest that TRALI occurs when donor plasma contains antibodies reactive against the recipient's HLA type or against recipient non-HLA leukocyte antigens. Some have postulated that TRALI may result when recipient leukocyte antibodies react with residual donor leukocytes, although solid evidence is lacking. However, an FNHTR reaction is more common in this setting. A second mechanism proposes a two-hit model involving lipid agents in donor blood that prime recipient neutrophils in the presence of specific cytokine activation.<sup>84</sup> Currently, TRALI is best documented to result from the passive transfer of antibodies directed against recipient leukocyte antigens. Thus, leukocyte reduction is not expected to play an important role in the prevention of TRALI.

### Transfusion-Associated Graft-vs-Host Disease

Transfusion-associated GVHD (TA-GVHD) results from the unchecked clonal expansion of allogeneic donor leukocytes (see Chapter 54). Patients who experience TA-GVHD either do not eliminate donor leukocytes because of severe immunosuppression or do not recognize donor cells as foreign because of HLA similarity between the donor and recipient. The threshold number of donor leukocytes required to provoke human TA-GVHD cannot be determined and likely varies among different donor-recipient pairs. Because TA-GVHD depends on recipient exposure to viable allogeneic donor leukocytes, it might be anticipated that sufficient leukocyte reduction would reduce the risk of TA-GVHD. However, there are no conclusive clinical data to support this notion, and TA-GVHD has been reported among isolated patients who received transfusion of LR blood components.<sup>85,86</sup> Therefore,  $\gamma$ -irradiation remains the only acceptable current means to prevent TA-GVHD, although emerging pathogen-reduction technologies may prove to be effective. Nevertheless, there are data that suggest the risk of TA-GVHD may be related to the residual leukocyte content of blood components. In an in-vitro study in which the mixed lymphocyte reaction was used to model GVHD, a logarithmic decline in the number of cells available to respond resulted in a decline in lymphocyte reactivity similar to that after irradiation.<sup>87</sup> Of greater in-vivo relevance, the number of instances in which blood is

transfused from a donor who is HLA homozygous for a haplotype shared by the recipient far exceeds the observed incidence of TA-GVHD. This finding, coupled with the observation that TA-GVHD is more common after transfusion of fresh blood components containing large numbers of leukocytes, suggests that under normal transfusion circumstances the decline in donor cell viability that occurs during routine blood storage may provide some degree of protection against TA-GVHD. A recent report describing the Serious Hazards of Transfusion data from Britain stated that the number of reported cases of TA-GVHD decreased from 12 to 1 before and after the introduction of universal leukocyte reduction in 1999, with no cases reported since 2001.<sup>88</sup> It is not clear whether this decline is a result of leukocyte reduction or other factors that changed over the same time frame, such as the age of the transfused red cells and/or more widespread use of irradiated blood components. The authors noted that the mean age of red cells at issue increased from 8 days to 12 to 14 days. Of potential relevance are new data showing that leukocyte reduction does not affect the rate at which long-term persistence of donor leukocytes (microchimerism) is observed after transfusion in trauma patients.<sup>89</sup> The important message is that leukocyte reduction may decrease, but does not eliminate, the risk of TA-GVHD.

### Transfusion-Related Immunomodulation

Allogeneic transfusions have been associated with a variety of immune phenomena, including tolerance to subsequent solid-organ transplantation, increased susceptibility to viral or bacterial infection, and diminished immune surveillance against tumors (see Chapter 45).<sup>90</sup> This effect has not been unequivocally linked to transfusion, and a large body of research has investigated a number of possible mechanisms. Early hypotheses included patient selection bias, clonal deletion of primed cells by immunosuppressive drugs, the development of idiotype antibodies, and the development of suppressor T cells. Subsequent proposals have included central thymic tolerance, effects of microchimerism, persistence of donor veto cells, shifting of T-cell cytokine expression, inhibition of natural killer cell activity,

and infusion of soluble HLA proteins or soluble mediators of apoptosis.

Several lines of evidence have suggested that recipient exposure to donor WBCs can result in improved survival of subsequent renal transplants. For example, the original studies of Opelz and Terasaki<sup>91</sup> showed that the use of frozen-deglycerolized (and thus leukocyte-reduced) blood did not protect against rejection of renal transplants as well as did other RBC preparations. Okazaki et al<sup>92</sup> subsequently found that the beneficial effect of pretransplantation transfusion could be obtained by means of transfusion of the buffy coat alone. In 1993, a prospective, double-blind, randomized trial showed that patients given postoperative transfusions of non-LR fresh RBCs had better renal allograft survival than did patients given frozen deglycerolized RBCs.<sup>93</sup> Because cyclosporine and tacrolimus have become widely adopted as immunosuppressive agents for organ transplantation, the contribution of transfusion to graft survival has declined. A more recent three-arm prospective RCT in 144 naïve patients undergoing cadaveric renal transplantation compared graft survival in patients who received pretransplant leukocyte-rich transfusions that were HLA-DR mismatched, those who received transfusions that were one HLA-DR antigen matched, and those who received no transfusions.<sup>94</sup> There was no difference in graft survival at 1 year or 5 years, indicating that there is no longer a routine role for pretransplant transfusions to prolong graft survival. Because the risk of HLA alloimmunization associated with using leukocyte-rich blood components outweighs any benefit from potential immune tolerance, LR components are indicated for organ transplant candidates.

Recipient exposure to allogeneic leukocytes has been proposed to account for increased susceptibility to bacterial infections among persons receiving transfusions. Numerous studies with human subjects have shown that postoperative infection is more common among patients who undergo transfusion than among patients who do not. However, such studies are flawed in that these two groups do not have comparable infectious risk. Thus, the observations do not shed light on the role of donor leukocytes or the value of leukocyte reduction. Unlike these studies, nine

**Table 16-8.** Randomized Controlled Trials of Leukocyte Reduction and Postoperative Infection\*

| Author          | Surgery            | Center Type and No. of Patients | LR Arm         | Control Arm | 95%CI of Relative Risk |
|-----------------|--------------------|---------------------------------|----------------|-------------|------------------------|
| Jensen          | Colorectal         | S197                            | LR Whole blood | Whole blood | 1.62-3.3*              |
| Houbiers        | Colorectal         | M697                            | LR RBCs        | Buffy       | 0.64-1.19*             |
| Jensen          | Colorectal         | S589                            | LR RBCs        | Buffy       | 2.16-5.20*             |
| van de Watering | Cardiac            | S909                            | LR RBCs        | Buffy       | 1.00-1.99*             |
| Tartter         | Gastrointestinal   | S221                            | LR RBCs        | RBCs        | 0.89-3.85*             |
| Wallis          | Cardiac            | S595                            | LR RBCs        | Buffy       | 0.6-1.9*               |
| Titlestad       | Colorectal         | S279                            | LR RBCs        | Buffy       | 0.9-4.0*               |
| Bilgin          | Cardiac            | M474                            | LR RBC         | Buffy       | 1.08-2.49*             |
| Van Hilten      | Aortic, colorectal | M1051                           | LR RBC         | Buffy       | 0.73-1.32              |

\*Data derived from Vamvakas.<sup>95</sup>

LR = leukocyte reduced; CI = confidence interval; S = single center; M = more than one center; RBCs = Red Blood Cells.

RCTs have been performed to examine postoperative infection rates among recipients of LR or non-LR blood (Table 16-8). These studies have provided conflicting results; some show benefit of leukocyte reduction and others do not. An “intention to treat” meta-analysis of eight studies (excluding the report of Van Hilten et al) indicated that there was no significant transfusion-related immunomodulation (TRIM) effect.<sup>95</sup> However when the three cardiac surgery studies were analyzed separately, a statistically significant TRIM effect was observed with a summary odds ratio (OR) of 1.39 (95% CI 1.08-1.80), indicating that cardiac surgery patients transfused with non-LR components had a 39% higher risk of postoperative infection. A subsequent meta-analysis of these eight studies and the additional RCT by van Hilten<sup>96</sup> included only those patients who were *actually transfused* and found a statistically significant ( $p=0.005$ ) OR of 0.52 across all studies, indicating a nearly 50% reduction in the risk of postoperative infection associated with the use of LR components.<sup>97</sup> Subset analysis indicated that the three studies in patients undergoing cardiac surgery were the primary contributors to the overall statistical significance. One limitation of these meta-analyses is that the studies differed in the type of component assigned to the non-LR control with some using standard red cells and others using buffy-coat-depleted red cells. Overall, however, these data increasingly suggest a beneficial role of LR blood in reducing the risk of postoperative infections, particularly in patients undergoing cardiac surgery.

Recipient exposure to donor leukocytes was previously considered possibly associated with an increased rate of relapse after resection of colorectal carcinoma. Preclinical studies with rodents demonstrated that the deliberate inoculation of tumor cells produced more pulmonary metastasis or tumor growth if followed by transfusion of blood that contained allogeneic leukocytes rather than transfusion of syngeneic or LR blood.<sup>98</sup> Although several clinical studies have shown that patients who undergo transfusion have a higher relapse rate than do patients who do not undergo transfusion, such studies cannot be used to draw conclusions about the effect of leukocyte reduction. In three RCTs with human subjects, investigators directly compared outcomes among patients receiving either LR or buffy-coat-depleted blood. These studies did not show a beneficial effect of leukocyte reduction.<sup>90</sup> In a 2001 randomized trial, investigators found no beneficial effect of leukocyte reduction on 5-year cancer recurrence rates among patients with colorectal carcinoma.<sup>99</sup> In light of current evidence, leukocyte reduction cannot be considered indicated for the prevention of a purported TRIM effect on cancer recurrence.

Leukocyte-reduced blood components have been reported to be associated with improved patient outcomes, specifically reduced mortality and/or hospital length of stay unrelated to postoperative infection. This effect has been primarily studied in cardiac surgery patients. A prospective study of three serial periods of transfusion strategies in cardiac surgery patients using non-LR components, LR components, and then non-LR components showed that hospital length of stay declined from 10.1 to 9.5 days with the use of LR components and then returned to 10.8 days with the reintroduction of non-LR components. There was no change in length of stay

in control patients who did not require transfusions.<sup>100</sup> A meta-analysis of three RCTs studying LR components in cardiac surgery showed that leukocyte reduction was associated with reduced mortality as well as a reduced risk of postoperative infection.<sup>101</sup> These data are supported by a recent prospective RCT of LR blood components in 562 patients undergoing cardiac surgery which found that those who received LR components had a significant reduction in mortality at 60 days (9.7 vs 4.9%,  $p = 0.029$ ) and at 1 year (11.7% vs 7.0%,  $p = 0.053$ ) compared to control patients.<sup>102,103</sup> The excess mortality was primarily caused by heart failure, because there was no difference in postoperative infection rates. Taken together these data provide convincing evidence in support of the use of LR components in patients undergoing cardiac surgery.

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## Adverse Effects of Leukocyte Reduction

Little evidence suggests a serious clinical disadvantage of leukocyte reduction. The technology is expensive—estimated at more than \$1 billion every 2 years for the United States alone. Concern has been raised that this cost will divert resources from other initiatives of greater consequence to safe transfusion practice.<sup>1</sup> In addition to the cost, three disadvantages have been observed.

First, filtration results in a substantial loss of the therapeutic blood element intended for transfusion. The VATS<sup>78</sup> found that patients randomly assigned to the leukocyte reduction arm received a statistically smaller mass of red cells.<sup>104</sup> It is unclear whether this actually results in additional red cell transfusions. This problem may be particularly important if filtration is combined with other manipulations, such as preparation of RBCs by the buffy coat removal technique, preparation of washed platelet concentrates, or pathogen inactivation techniques.

Second, bedside leukocyte reduction has been associated with hypotensive reactions, especially among recipients treated with ACE inhibitors.<sup>25</sup> As blood passes through negatively charged filters, contact activation occurs. The activation, in turn, causes generation of short-lived vasoactive proteins such as bradykinin, leading to hypotension. For example, Takahashi et al<sup>105</sup> found that some filters were associated with the production of bradykinin during filtration of platelet concentrates. Bradykinin level increased from 37 pg/mL to 6794 pg/mL as a result of filtration. If an ACE inhibitor was added to the plasma, the level increased to 36,000 pg/mL because the ACE inhibitor also inhibits the kininases responsible for the breakdown of bradykinin. These reactions led to recommendations to use prestorage leukocyte reduced components to eliminate this complication.<sup>106</sup> Recently, these reactions were reported in a patient taking ACE inhibitors despite the fact that the blood was prestorage leukocyte reduced; an inherited defect in kinin metabolism was suspected.<sup>107</sup> Thus, patients taking ACE inhibitors and patients with inherited deficiencies of kininases are more susceptible to the hypotensive effects of administered bradykinin.

Third, reports of allergic reactions characterized by acute conjunctivitis, pain at the site of blood infusion,<sup>108</sup> or the sudden

onset of back pain and hypertension<sup>109</sup> have been observed in some patients undergoing transfusion of LR blood. Although these events appear to occur in a very small proportion of patients who undergo transfusion, the studies highlight that even low-frequency adverse consequences attributed to leukocyte filtration will affect growing numbers of patients as the technology becomes more widely used.

## Universal Leukocyte Reduction

Nearly all of Europe and Canada have implemented leukocyte reduction of all cellular blood components—termed “universal leukocyte reduction.” It has not been mandated by the FDA in the United States and the most recent survey data indicate that 72% of red cells, 40% of whole-blood-derived platelets, and nearly all apheresis platelets are leukocyte reduced.<sup>30</sup> These percentages may reflect availability more than clinical preference. In October 2000, the University Health Consortium concluded that the available evidence was insufficient to recommend universal leukocyte reduction. In contrast, the US Blood Safety and Availability Committee recommended in January 2001 that universal leukocyte reduction be implemented as “soon as is feasible.” Adoption of universal leukocyte reduction remains a hotly debated topic.<sup>110</sup> A prospective RCT of leukocyte reduction in a major academic medical center failed to demonstrate a difference in in-hospital mortality, hospital length of stay, or other secondary outcomes.<sup>111</sup> Other RCTs in HIV-infected patients<sup>78</sup> and trauma patients<sup>112</sup> failed to show a benefit in patient outcomes. A “before and after” study of the effects of adopting universal leukocyte reduction in Canada reported that the unadjusted hospital mortality rates declined from 7.03% to 6.19% ( $p < 0.04$ ).<sup>113</sup> There was no difference in serious nosocomial infections, although posttransfusion fevers and antibiotic use did decrease. A similar analysis of Canadian data in premature neonates found no difference in the primary outcomes: nosocomial bacteremia and neonatal intensive care unit mortality.<sup>114</sup> Improvement in some secondary clinical outcomes were reported. These two studies must be interpreted with caution, however, because of their retrospective design and potential bias from confounders. When viewed in the context of the negative RCTs noted above, adoption of universal leukocyte reduction cannot be justified on the basis of available scientific evidence. Other considerations such as logistics, public policy, public perception, and mechanisms for funding leukocyte reduction all play a role in making a final decision regarding adoption of universal leukocyte reduction.

## Summary

The use of LR cellular blood components has expanded dramatically in the last decade. The development of high-performance blood filters and low-leukocyte apheresis devices have made LR components available to all transfusion facilities.

The technology represents an important advance in the preparation of blood components. Several European nations and Canada have adopted universal leukocyte reduction of the blood supply. However, leukocyte reduction represents the largest incremental increase in the cost of producing blood components in history. Although billions of dollars in health-care resources would be needed to implement universal leukocyte reduction in the developed world, clinical trials have shown benefit for only limited patient populations.

## Disclaimer

D. Triulzi has disclosed financial relationships with ZymeQuest and Cerus. W. Dzik has disclosed no conflicts of interest.

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## 17

# Composition and Mechanisms of Blood Coagulation and Fibrinolysis

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The first part of this chapter describes the composition of plasma, focusing on those protein components that are important for treatment with plasma or its derivatives. The second part addresses individual factors influencing plasma levels of therapeutic plasma proteins. The regulation of blood coagulation and fibrinolysis and clinically useful blood coagulation and fibrinolysis screening tests are then discussed.

## Plasma Composition

Plasma is the cell-free part of blood composed of water, proteins, electrolytes, lipids, and carbohydrates. It is the transportation medium through which nutrients, hormones, waste products, and drugs are transported through the body. In vivo, the fluidity of plasma is maintained through complex interactions between its procoagulant and anticoagulant proteins and between these proteins, circulating blood cells, and the endothelium. Coagulation activation in plasma is prevented and its fluidity maintained in vitro by the addition of anticoagulants. Citrate is presently the only anticoagulant used for collecting therapeutic plasma and plasma for fractionation, the starting material for the manufacture of plasma protein concentrates. Although proteomics based on two-dimensional gel electrophoresis and mass spectrometry has revealed about 10,000 different human plasma proteins,<sup>1,2</sup> the number of constituents important for transfusion medicine is small, comprising albumin, immunoglobulins G (IgG) and M (IgM),  $\alpha_1$ -antitrypsin, C1 inhibitor, von Willebrand factor (vWF)-cleaving protease, blood coagulation factors, vWF, and the blood coagulation inhibitors antithrombin (AT) and protein C. The plasma levels and biological half-lives of these plasma proteins are subject to large interindividual

variation. Plasma is either prepared from citrate-phosphate-dextrose-anticoagulated whole blood units or collected by automated plasmapheresis. As a consequence of lower final citrate concentrations and lower dilution due to lower anticoagulant-to-blood ratios, apheresis plasma contains higher concentrations of clotting Factors V, VIII, IX, and XI than plasma from whole blood.<sup>3,4</sup> A dilution of 1:7 to 1:9 for plasma from whole blood and of 1:16 for apheresis plasma has to be considered when plasma protein concentrations are given that are related to circulating undiluted plasma. IgG levels in plasma from whole blood are significantly greater than in apheresis plasma because of a decrease in IgG when individuals donate apheresis plasma repeatedly at short intervals.<sup>4,5</sup>

## Albumin

Albumin is a 66.4-kD protein synthesized in the liver. Considering a total plasma volume of 3 L, about 120 to 140 g or 40% of the total body content of albumin circulates in the plasma at concentrations between 35 and 50 g/L (Table 17-1), which is more than 50% of the total plasma protein concentration, ranging from 64 to 88 g/L. The albumin concentration in the interstitial space of 10 to 12 L is about 14 g/L. The intravascular albumin compartment is responsible for 80% of plasma oncotic pressure, thus mainly contributing to maintaining blood volume. An intact liver synthesizes 12 g of albumin per day. In a steady state, the same amount of albumin is eliminated per day. The biological half-life of albumin in plasma is 17 to 20 days. Compensatory reduction of albumin metabolism occurs when plasma levels decrease because of reduced synthesis in the liver or capillary leakage.<sup>6</sup> A marked acquired hypoalbuminemia is a nonspecific marker of severe illness and strongly indicates poor prognosis.<sup>7,8</sup>

The functions of albumin are as follows:

- Maintenance of the intravascular oncotic pressure.
- Binding and transportation of drugs, vitamins, minerals, bilirubin, uric acid, hormones, amino acids, and fatty acids.
- Binding of toxic metabolites, among them free fatty acids, and radical scavenging.



**Table 17-1.** Therapeutic Plasma Proteins: Albumin, Immunoglobulins, Alpha-<sub>1</sub>-Antitrypsin, and C1 Inhibitor

| Plasma Protein                   | Plasma Concentration | Biological Half-Life | Main Functions                                                                           |
|----------------------------------|----------------------|----------------------|------------------------------------------------------------------------------------------|
| Albumin                          | 35-50 g/L            | 17-20 days           | Maintenance of intravascular oncotic pressure; transportation protein; radical scavenger |
| Immunoglobulin G                 | 6-17 g/L             | 23 days              | Humoral immunity                                                                         |
| Immunoglobulin M                 | 0.5-2.5 g/L          | 5 days               | Humoral immunity                                                                         |
| Alpha- <sub>1</sub> -antitrypsin | 1.5-3.0 g/L          | 4-7 days             | Inhibits neutrophil elastase                                                             |
| C1 inhibitor                     | 0.2-0.3 g/L          | 30-60 hours          | Inhibits complement C1 activation                                                        |

Plasma concentrations represent the 5th and 95th percentiles; biological half-lives represent the means or the 5th and 95th percentiles of elimination half-lives.

Despite all these important functions of albumin, it is remarkable that congenital analbuminemia causes only mild clinical symptoms (such as hypotension or discrete pretibial edema at all ages) or no symptoms at all.<sup>9</sup>

Commercially available albumin preparations are obtained from plasma fractionation according to Cohn and Oncley<sup>10</sup> or Kistler and Nitschmann<sup>11</sup> as fraction V and subsequently pasteurized for virus inactivation.<sup>12</sup> These solutions contain either 3.5% to 5% or 20% to 25% (w/v) of albumin at a purity of 95% to 98%. Albumin is also added to many drugs and plasma protein concentrates to stabilize the respective active ingredients.

**Immunoglobulins G and M**

IgG and IgM are glycoproteins synthesized in plasma cells and circulating in plasma at concentrations between 6 and 17 g/L and between 0.5 and 2.5 g/L, respectively. The molecular weights are 150 kD for IgG and 971 kD for IgM and the half-lives are about 23 and 5 days, respectively.

Polyvalent intravenous immunoglobulin (IVIG) preparations can also be administered subcutaneously and are prepared from fraction II during plasma fractionation.<sup>12</sup> Current virus inactivation and elimination procedures are low pH incubation, pasteurization, solvent/detergent treatment, caprylic acid treatment, and nanofiltration. While most IVIG preparations are primarily IgG with only trace amounts of other immunoglobulin isotypes, some IgM-enriched IVIGs contain up to 15% IgM and have been used for treatment of severe sepsis and septic shock.<sup>13</sup>

Hyperimmune immunoglobulin preparations are obtained from the plasma of actively immunized donors using ethanol fractionation or chromatography.<sup>12</sup> They can be administered intravenously or intramuscularly and are used to prevent postexposure infections, intoxication by tetanus toxoid, and immunization to Rh-positive red cells. For more details see Chapter 18.

**Alpha-<sub>1</sub>-Antitrypsin**

Alpha-<sub>1</sub>-antitrypsin synthesized in the liver is the most abundant serine protease inhibitor (serpin) in plasma. It inhibits a variety of serine proteases of which neutrophil elastase is the most important target. The molecular weight is 52 kD, the plasma concentration is 1.5 to 3 g/L, and the half-life is 4 to 7 days.<sup>14,15</sup> Hereditary alpha-<sub>1</sub>-antitrypsin deficiency resulting in plasma levels lower than 0.8 g/L occurs at a prevalence between 1 in 1600 to 1 in 5000.<sup>16</sup> It is associated with lung emphysema and liver disease in childhood. Plasma-derived alpha-<sub>1</sub>-antitrypsin concentrates are indicated for treatment of patients with emphysema secondary to congenital deficiency.

**C1 Inhibitor**

C1 inhibitor is a 105-kD serpin synthesized in the liver. The plasma concentration is 0.24 g/L, and the half-life ranges from 30 to 60 hours.<sup>17,18</sup> C1 inhibitor affects early activation of the classical complement pathway by blocking activated C1 and subsequent C2,4 complex formations.<sup>19</sup> It also inhibits activated Factors XI and XII and plasmin, and the formation of Factor XIa, kallikrein, and bradykinin.

An inherited or acquired deficiency of C1 inhibitor may cause angioedema characterized by subcutaneous or mucosal swelling in any part of the skin or the respiratory and gastrointestinal tracts.<sup>19</sup> The prevalence of hereditary angioedema is 1 in 10,000 to 1 in 50,000. C1 inhibitor concentrates purified by chromatography from the cryoprecipitate-reduced plasma produced during plasma fractionation are used successfully for the treatment of acute attacks of angioedema or for prophylaxis before surgery.<sup>17,20</sup>

**von Willebrand Factor Cleaving Protease**

The vWF-cleaving protease (ADAMTS13) is a 70-kD protein<sup>21</sup> expressed in the liver, platelets, and other tissues,<sup>22,23</sup> and circulates in plasma at concentrations between 0.7 and 1.3 mg/L.<sup>24</sup> The elimination half-life is 2 to 3 days.<sup>25</sup> The plasma enzyme cleaves the A2-domain of vWF. A defective cleaving protease results in unusually large vWF multimers, which are associated with thrombotic thrombocytopenic purpura (TTP). Therapeutic plasma contains normal vWF-cleaving protease activities and is effective for treatment of TTP through therapeutic plasma exchange.<sup>26,27</sup>

**Clotting Factors, Coagulation Factor Inhibitors, and von Willebrand Factor**

The coagulation proteins discussed here are responsible for normal hemostasis (Table 17-2). Except for Factor V, virus-inactivated plasma-derived concentrates have been developed for preventing or treating bleeding caused by hereditary or acquired vWF or any clotting factor deficiency states.<sup>12</sup> Antithrombin and protein C concentrates are also available and are used mainly for prophylaxis or treatment of thromboembolic complications in patients with hereditary deficiency of the respective blood coagulation inhibitor.<sup>12,49,50</sup> Because not all clotting factor and inhibitor concentrates are available in all developed countries, Factor V deficiency, for which a concentrate has not yet been produced,

**Table 17-2.** Clotting Factors, von Willebrand Factor, Antithrombin, and Protein C

| Plasma Protein | MW (kD)                 | Plasma Concentration (mg/L) | Plasma Activity (U/dL)  | Half-Life (hours)   | Main Functions                                                                                                  | Replacement by Plasma-Derived Concentrates <sup>12</sup> or Plasma                     |
|----------------|-------------------------|-----------------------------|-------------------------|---------------------|-----------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------|
| Fibrinogen     | 340                     | 2.5 g/L                     | 1.5-4 g/L <sup>28</sup> | 96 <sup>28</sup>    | Clot formation, precursor of fibrin; supports platelet function                                                 | Fibrinogen concentrate, cryoprecipitate, plasma; locally, fibrin sealant <sup>29</sup> |
| Factor II      | 72                      | 150 <sup>30</sup>           | 70-130                  | 60 <sup>31,32</sup> | Proenzyme of thrombin                                                                                           | Prothrombin complex concentrates (PCC), plasma <sup>28,31</sup>                        |
| Factor V       | 330                     | 7 <sup>33,34</sup>          | 70-125                  | 12 <sup>35</sup>    | Cofactor of prothrombin activation by Factor Xa                                                                 | Plasma <sup>28</sup>                                                                   |
| Factor VII     | 50                      | 1 <sup>36</sup>             | 70-150                  | 5 <sup>31,27</sup>  | Proenzyme of Factor VIIa, which activates Factor IX and Factor X                                                | Factor VII concentrates, Factor VII containing PCC                                     |
| Factor VIII    | 330                     | 0.15 <sup>38</sup>          | 60-200                  | 14 <sup>39</sup>    | Cofactor of Factor X activation by Factor IXa                                                                   | Factor VIII concentrates, Factor VIII/vWF concentrates                                 |
| vWF            | 600-2 × 10 <sup>4</sup> | 8 <sup>40</sup>             | 50-180                  | 12 <sup>41,42</sup> | Platelet-subendothelium adhesion; platelet aggregation; transports Factor VIII and protects it from proteolysis | Factor VIII/vWF concentrates, vWF concentrates                                         |
| Factor IX      | 57                      | 5 <sup>40</sup>             | 60-140                  | 30 <sup>39</sup>    | Proenzyme of Factor IXa, which activates Factor X                                                               | Factor IX concentrates                                                                 |
| Factor X       | 59                      | 10 <sup>40</sup>            | 70-130                  | 30 <sup>31</sup>    | Proenzyme of Factor Xa, which activates prothrombin                                                             | PCC, plasma <sup>28</sup>                                                              |
| Factor XI      | 143                     | 5 <sup>43</sup>             | 65-130                  | 50 <sup>44,45</sup> | Proenzyme of Factor XI, which activates Factor IX                                                               | Plasma, Factor XI concentrates <sup>28,43</sup>                                        |
| Factor XIII    | 320                     | 20 <sup>46</sup>            | 60-220                  | 170 <sup>28</sup>   | Proenzyme of Factor XIIIa, which stabilizes fibrin by cross-linkage                                             | Factor XIII concentrates                                                               |
| Antithrombin   | 58                      | 150 <sup>47</sup>           | 75-120                  | 72 <sup>47</sup>    | Stoichiometric serpin, inactivates thrombin, Factor IXa, Factor Xa, and Factor XIIa                             | Antithrombin concentrates <sup>49</sup>                                                |
| Protein C      | 62                      | 44 <sup>40</sup>            | 70-170                  | 9 <sup>48,49</sup>  | Proenzyme of activated protein C, which inactivates Factor Va and Factor VIIIa                                  | Protein C concentrates <sup>50</sup>                                                   |

Plasma concentrations represent the means or medians; plasma activities represent the 5th and 95th percentiles; half-lives represent the mean or median elimination half-lives; PCC = prothrombin complex concentrate; vWF = von Willebrand factor.

and other clotting factor deficiency states (eg, Factor XI deficiency) are alternatively treated with plasma.<sup>28</sup>

Fibrinogen is the precursor of fibrin. Thrombin converts fibrinogen into fibrin monomers by cleaving fibrinopeptides A and B. Fibrin monomers polymerize to form an unstable fibrin clot, which is stabilized by Factor XIIIa-induced cross-linkage. Fibrinogen is also necessary for supporting platelet function. Prothrombin (Factor II) and clotting Factors VII, IX, X, and XI, and protein C are proenzymes of respective serine proteases. After activation by thrombin, Factor V accelerates activation of prothrombin, Factor VIII, and Factor X. Factor XIII is activated by thrombin to form a transglutaminase, which stabilizes fibrin clots by cross-linkage. vWF is essential for platelet-subendothelium adhesion and platelet aggregation, and for transportation of Factor VIII in plasma. vWF protects Factor VIII from early proteolysis. Antithrombin is a stoichiometric serpin that strongly inhibits thrombin and Factors IXa, Xa, and XIa. Once activated by thrombin-thrombomodulin, activated protein C inhibits Factor Va and Factor VIIIa.

Most clotting factors, antithrombin, and protein C are synthesized in the liver. Other sites of protein expression are megakaryocytes and endothelial cells for Factor V and for the  $\alpha$ -chain of Factor XIII. vWF is synthesized in megakaryocytes and endothelial cells.

The synthesis of functionally active prothrombin complex Factors II, VII, IX, and X, and protein C in the liver requires vitamin-K-mediated gamma-glutamyl carboxylation. Impairment of carboxylation occurs in vitamin K deficiency, in the presence of vitamin K antagonists, and in some cases of liver dysfunction. This results in synthesis of functionally impaired or inactive prothrombin complex factors, called PIVKA or proteins induced in vitamin K absence or antagonism.

## Factors Influencing Individual Plasma Composition

### Age and Gender

The physiologic aging process is accompanied by an increase in plasma levels of fibrinogen, Factors VII, VIII, and IX, and protein C in both genders.<sup>51</sup> A significant decrease of prothrombin activity levels with increasing age has been found in men but not in women.<sup>52</sup> Young women have significantly lower AT plasma levels than males of similar age. Because of a marked increase after menopause, AT levels in older women exceed levels in male contemporaries.<sup>53</sup> Gradually declining AT plasma levels have been observed in males older than 45 years.<sup>53</sup> Pregnancy is associated with marked increases in levels of fibrinogen, prothrombin, and Factors VII, VIII, and X, whereas Factor V, Factor IX, AT, and protein C levels are unchanged.<sup>54</sup>

### ABO Blood Group

Individuals with blood groups O, A<sub>2</sub>O, and A<sub>2</sub>A<sub>2</sub> have on average about 20 to 25% lower Factor VIII and vWF plasma levels when compared with other ABO blood group constellations.<sup>55-57</sup>

### Acute Phase Reaction

An acute phase response caused by any trigger of inflammation results in an increase in plasma levels of  $\alpha_1$ -antitrypsin, C1

inhibitor, fibrinogen, prothrombin, Factor VIII, and vWF and a decrease of albumin levels.<sup>58</sup> Smoking causes a low-grade systemic inflammatory response and concomitant increases in plasma fibrinogen.<sup>59-61</sup>

## Physical Exercise and Mental Stress

Both acute severe physical exercise and acute mental stress cause increases in fibrinogen, Factor VII, Factor VIII, and vWF levels.<sup>63-66</sup> In contrast, prolonged severe physical exercise results in a decrease in Factor VII levels, while fibrinogen levels continue to be increased during observation.<sup>68</sup>

## Hormones

Danazol, a weak androgen, improves the synthesis of C1 inhibitor, Factor VIII, AT, and protein C.<sup>68</sup> Oral contraceptives induce increases in plasma fibrinogen, prothrombin, Factors VII, VIII, IX, X, and XI,  $\alpha_1$ -antitrypsin, and protein C, while AT and protein S levels decrease.<sup>69-71</sup> Androgenic progestogens contained in oral contraceptives, but not progestogens with less androgenic profile, attenuate the ethinylestradiol-induced changes in hemostasis.<sup>72</sup>

## DDAVP

Desmopressin (DDAVP) has been used successfully to increase Factor VIII and vWF levels in plasma donors.<sup>73,74</sup> The yields of these plasma proteins were markedly improved when cryoprecipitate or Factor VIII concentrates were produced from plasma collected after pretreatment of donors with DDAVP.

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## Regulation of Blood Coagulation and Fibrinolysis

Hemostasis encompasses all processes of clot formation, clot lysis, and wound healing with the aim of maintaining vessel wall integrity. The mechanisms of hemostasis incorporate the vascular endothelium and subendothelium, platelets, leukocytes, red cells, pro- and anticoagulant, and pro- and antifibrinolytic proteins. Primary hemostasis is characterized by the formation of a platelet plug at the site of vessel wall injury. vWF forms a bridge between platelets and vascular subendothelial structures and promotes platelet aggregation. Secondary hemostasis is achieved by formation of a stable fibrin clot, followed by fibrinolysis and tissue repair. Abnormal bleeding can result from impaired thrombin generation, clot formation, and clot stabilization, or from hyperfibrinolysis. Conversely, increased coagulation and diminished fibrinolysis may provoke thrombosis.

Previous models have suggested two independent pathways of coagulation activation, via the intrinsic and extrinsic cascade. But it is now recognized that the generation or exposure of tissue factor (TF) at the site of vessel injury is the first physiologic step in initiating blood coagulation.<sup>75</sup> There are three closely linked phases of coagulation, comprising the initiation, amplification, and propagation processes (Fig 17-1). Clotting is mainly controlled by several circulating inhibitors. Activation of fibrinolysis

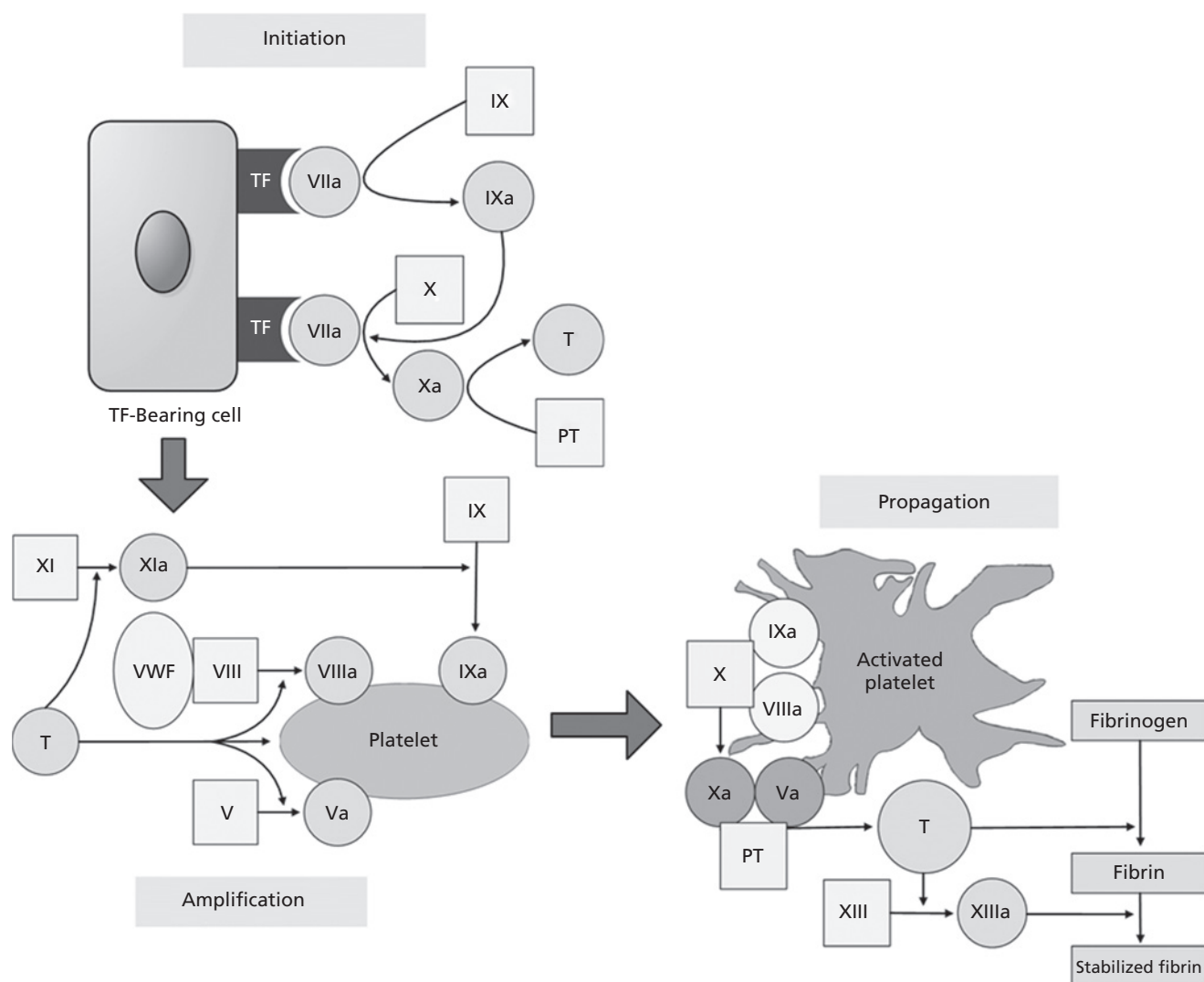


**Figure 17-1.** Cell-based model of coagulation activation. Inactive clotting factors are in squares and active factors in circles. The coagulation cascade is initiated by tissue factor (TF) release at the sites of cell injury. During the initiation phase, TF complexes with Factor VIIa (FVIIa) at the site of cell injury. TF-Factor VIIa activates Factor IX and Factor X. Factor Xa generates small amounts of thrombin, which activates platelets, Factor XI, and cofactors V and VIII, allowing multiple positive feedback loops during the amplification phase. The tenase complex (Factor VIIIa-Factor IXa) generates large amounts of Factor Xa on the surface of activated platelets. The prothrombinase complex (Factor Va-Factor Xa) converts prothrombin to thrombin, which forms fibrin from fibrinogen and activates Factor XIII. PT = prothrombin; T = thrombin; TF = tissue factor.

results in fibrin cleavage by plasmin and subsequent restoration of vessel patency. The central role of thrombin in hemostasis by exerting both procoagulant and anticoagulant effects is illustrated in Fig 17-2.<sup>76</sup>

### Initiation and Amplification of Coagulation

Clotting is initiated by TF-Factor VIIa complexes after TF is exposed to the circulating blood by smooth muscle cells, fibroblasts, disrupted or activated endothelial cells, or activated monocytes (Fig 17-1). Circulating Factor VIIa represents approximately 1% of total Factor VII and can be generated from the inactive zymogen by an autocatalytic mechanism. Factor VIIa does not express its full procoagulant activity until it is bound to TF.<sup>78</sup>

TF-Factor VIIa activates Factors IX and X, and Factor Xa generates thrombin by activating prothrombin (*initiation phase*). These small amounts of thrombin ensure that coagulation activation and further thrombin formation are maintained (*amplification phase*). Thrombin activates Factors V, VIII, and XI, and platelets that provide the surface on which the *propagation phase* of coagulation occurs.

### Propagation of Thrombin Generation, Fibrin Clot Formation, and Fibrin Stabilization

The *tenase complex* consisting of platelet-membrane-bound Factor IXa, Factor VIIIa, and ionized calcium cleaves Factor X much more effectively than TF-Factor VIIa or Factor IXa alone.

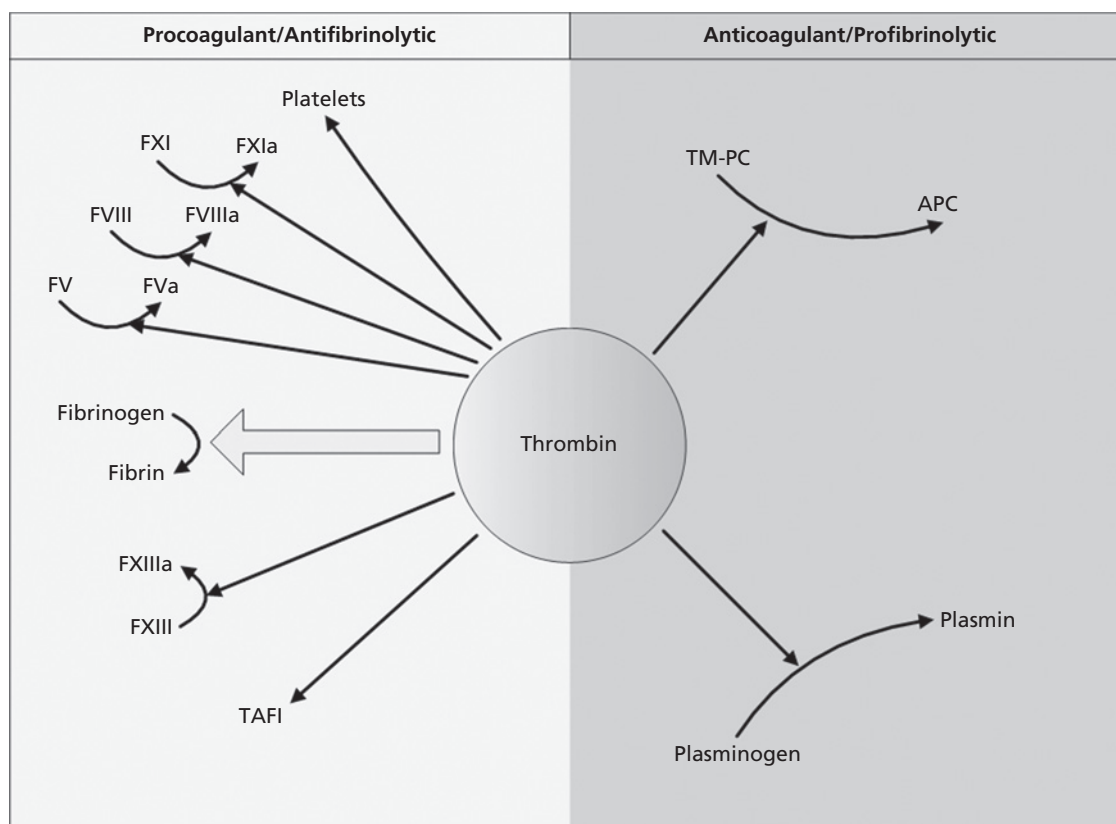


**Figure 17-2.** The central role of thrombin in hemostasis. Procoagulant effects include platelet activation, the activation of clotting Factors V, VIII, XI, and XIII, and subsequent formation of a stable fibrin clot. Thrombin acts as an anticoagulant by activating protein C after complexing with thrombomodulin. It promotes fibrinolysis by activating plasminogen and inhibits fibrinolysis by activating TAFI. APC = activated protein C; F = factor; PC = protein C; TAFI = thrombin activatable fibrinolysis inhibitor; TM = thrombomodulin.

Factor Xa binds to platelet surfaces and complexes with Factor Va and calcium to form the *prothrombinase complex*, which rapidly generates large amounts of thrombin. Fibrin monomers resulting from thrombin-induced splitting of fibrinopeptides A and B from fibrinogen polymerize spontaneously to an unstable fibrin clot. Thrombin also activates Factor XIII to the transglutaminase Factor XIIIa, which stabilizes fibrin polymers by cross-linkage.<sup>78</sup>

### Inhibition of Blood Coagulation and Termination of Clotting

Several circulating inhibitors terminate the coagulation process (Table 17-3). The stoichiometric heparin-binding serpin AT mainly inhibits thrombin and Factor Xa and less strongly Factors IXa and XIa.<sup>47</sup> Thrombin binds to endothelial thrombomodulin and this complex activates protein C to the serin protease-activated protein C (APC).<sup>79</sup> Endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. Together with its vitamin-K-dependent cofactor protein S, APC inactivates Factors Va and VIIIa. Tissue factor pathway inhibitor (TFPI) down-regulates blood coagulation by forming a quaternary complex with TF-Factor VIIa/Factor Xa. Heparin cofactor II is a specific inhibitor of thrombin in the presence of dermatan sulfate or heparin.<sup>47</sup>

The protein-Z-dependent protease inhibitor is a serpin inhibiting Factor Xa in the presence of the vitamin-K-dependent protein Z, phospholipids, and calcium.<sup>47</sup> Protein C inhibitor, a heparin-binding serpin, predominantly inhibits APC and thrombin.<sup>47</sup>

### Fibrinolysis and Its Inhibition

The role of the fibrinolytic system is to restore vessel patency once wound healing has maintained vessel wall integrity (Fig 17-3). The serin protease plasmin cleaves fibrin (and fibrinogen) at multiple sites, resulting in fibrin and fibrinogen degradation products. Plasminogen is activated to plasmin by tissue plasminogen activator (tPA) released by endothelial cells or by twice-chain urokinase plasminogen activator (tcuPA; urokinase) resulting from the activation of single-chain urokinase plasminogen activator (scuPA), which is synthesized in endothelial and kidney cells.<sup>80</sup> Besides plasmin, Factor VII activating protease is a potent activator of scuPA.<sup>81</sup> Plasmin cleaves not only fibrin but also fibrinogen and other plasma proteins. Fibrin specificity of plasmin is achieved by binding of plasminogen and tPA to the lysine binding sites of fibrin. Both plasminogen activators are mainly inhibited by plasminogen activator inhibitor type 1 (PAI-1) released from endothelial cells and platelets. Large

**Table 17-3.** Inhibitors of Blood Coagulation and Their Main Functions

| Plasma Protein                         | Main Functions                                                                                                                 |
|----------------------------------------|--------------------------------------------------------------------------------------------------------------------------------|
| Antithrombin                           | Heparin-binding serpin; inhibits thrombin, Factor Xa, Factor IXa, Factor XIa                                                   |
| Protein C                              | Serine protease; inhibits Factor Va and Factor VIIIa                                                                           |
| Protein S                              | Vitamin-K-dependent glycoprotein; cofactor of activated PC                                                                     |
| Thrombomodulin                         | Endothelial cell-surface glycoprotein; complexes with thrombin and activates protein C                                         |
| Endothelial cell protein C receptor    | Endothelial cell-specific transmembrane protein; augments protein C activation                                                 |
| Tissue factor pathway inhibitor (TFPI) | Trivalent protease inhibitor; inhibits TF-Factor VIIa by forming a quarternary complex of TFPI, TF, Factor VIIa, and Factor Xa |
| Heparin cofactor II                    | Dermatan sulphate-binding serpin; inhibits thrombin                                                                            |
| Protein-Z-dependent protease inhibitor | Serpin; inhibits Factor Xa in the presence of protein Z                                                                        |

amounts of plasminogen activator inhibitor type 2 (PAI-2) are synthesized in the placenta. Plasmin is predominantly inhibited by the very fast-acting plasmin inhibitor ( $\alpha_2$ -antiplasmin) and to a lesser extent by  $\alpha_2$ -macroglobulin. Plasma inhibitor cross-linked to fibrin by Factor XIIIa inhibits plasmin binding to fibrin. The thrombin-activatable fibrinolysis inhibitor is activated by thrombin-thrombomodulin complexes and protects clots from plasmin-induced degradation by removing lysine residues from fibrin.<sup>82,83</sup>

### Clinically Useful Coagulation and Fibrinolysis Screening Tests

Coagulation and fibrinolysis screening tests are used for detecting disorders of secondary hemostasis caused by coagulation factor deficiencies, dysfunctional coagulation factors, inhibitors against coagulation factors, anticoagulants, or hyperfibrinolysis. In addition to a detailed clinical history and examination and

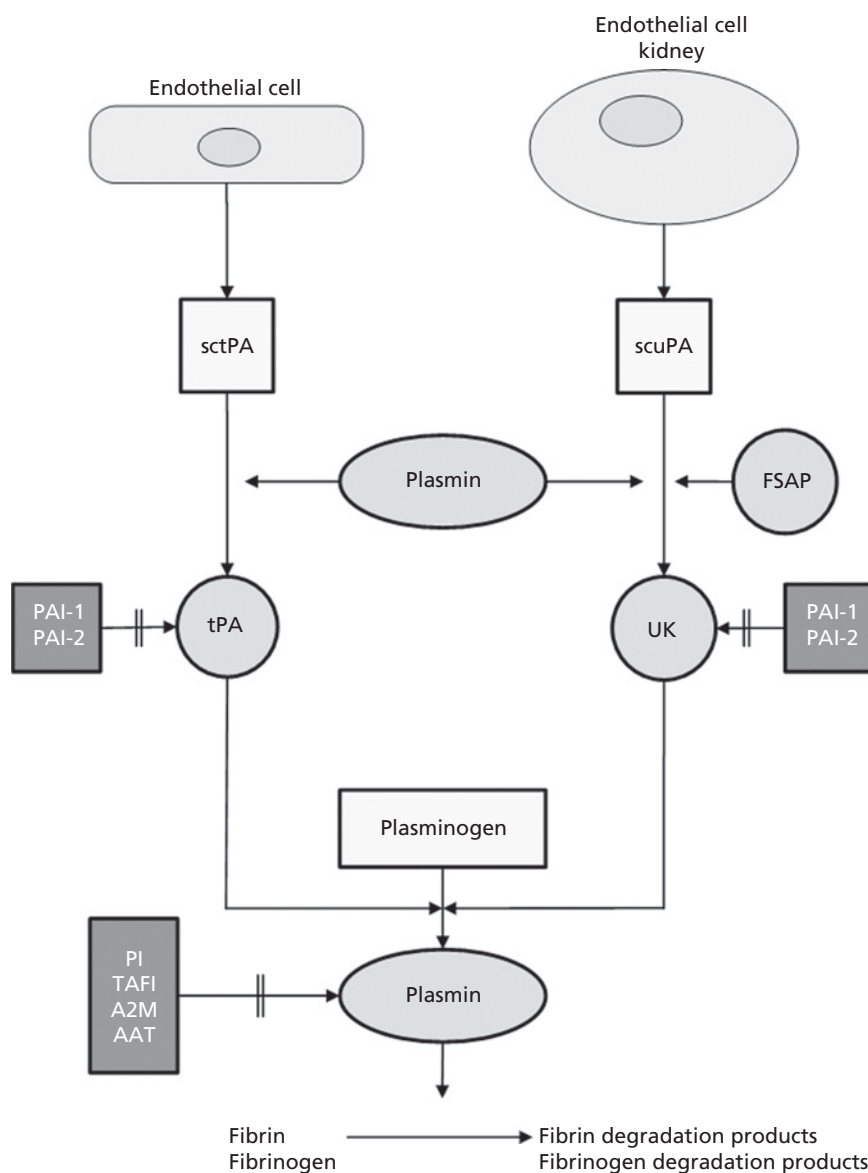


**Figure 17-3.** Activation of fibrinolysis. Endothelial cells release sctPA or scuPA, which are activated by plasmin to tPA and urokinase, respectively. scuPA can also be activated to urokinase by FSAP. Both activators are able to generate plasmin from plasminogen. Plasmin cleaves fibrin and fibrinogen to degradation products. tPA and urokinase are inhibited by PAI-1 and PAI-2 and plasmin by PI, A2M, and AAT. TAFI inhibits plasmin-induced cleavage of fibrin. AAT =  $\alpha_1$ -antitrypsin; A2M =  $\alpha_2$ -macroglobulin; FSAP = Factor VII activating protease; PAI-1 = plasminogen activator inhibitor 1; PAI-2 = plasminogen activator inhibitor 2; PI = plasmin inhibitor; sctPA = single-chain tissue plasminogen activator; scuPA = single-chain urokinase plasminogen activator; TAFI = thrombin activatable fibrinolysis inhibitor; tPA, = tissue plasminogen activator; UK = urokinase.

laboratory investigation of disorders of primary hemostasis, coagulation and fibrinolysis screening tests are included in approaches to bleeding patients and to exclusion of bleeding disorders before operations or other invasive procedures.<sup>84</sup> Further applications of these tests are as follows:

- Monitoring of anticoagulant therapy.
- Monitoring of treatment with fresh frozen plasma or clotting factor concentrates.
- Detection of lupus anticoagulants.
- Detection of hypercoagulable states.

The historical division of coagulation activation and subsequent fibrin formation into an *intrinsic pathway* comprising surface-contact factors and an *extrinsic pathway* (TF-Factor VIIa) can be used for a better understanding of blood coagulation screening tests (Fig 17-4). The intrinsic pathway begins with the activation of Factor XII and prekallikrein at negatively charged artificial surfaces, as found on activated platelets and endothelial cells and at subendothelial collagen. Factor XIIa activates Factor XIa and further prekallikrein to kallikrein. Factor XIa and kallikrein accelerate Factor XII activation through a positive feedback mechanism. Factor XII and Factor XI activation is amplified by high-molecular-weight (HMW) kininogen. Factor XIa activates Factor IX and Factor IXa activates Factor X. The extrinsic pathway starts with the formation of TF-Factor VIIa complex, also resulting in production of Factor Xa.

The common pathway begins with Factor X activation, where the intrinsic and extrinsic pathways converge. Factor Xa converts prothrombin (Factor II) to thrombin (Factor IIa) in the presence of Factor Va. Thrombin cleaves fibrin to form fibrin monomers, which polymerize spontaneously to an unstable fibrin clot.

Coagulation screening tests use fibrin polymerization as a measuring signal and have limited sensitivity to detect mild coagulation factor deficiencies or weak inhibitors against coagulation factors. Their specificity is also limited by the fact that several causes of abnormal clotting times are not associated with bleeding or thrombosis. Therefore, coagulation screening tests must be combined with the clinical history and symptoms when assessing the individual risk of bleeding or thrombosis.<sup>84-87</sup> Laboratories performing coagulation screening tests must consider the different sensitivities of reagents to clotting factor deficiencies, therapeutic anticoagulants, or lupus anticoagulants. Each laboratory should establish its own reference ranges, which depend not only on the reagents but also on the instruments used for analyses. It is also important to define a grey area of borderline assay results that should prompt specific coagulation assays despite a lack of clearly abnormal screening test results.

The *activated partial thromboplastin time* (aPTT) measures the intrinsic and common pathways of coagulation activation. The recalcification clotting time of citrated plasma is determined after addition of kaolin, celite, micronized silica, or ellagic acid, providing a large artificial surface area, and phospholipids functioning as a platelet substitute. The *prothrombin time* (PT) is performed by adding brain tissue or recombinant thromboplastin and calcium to citrated plasma and recording the clotting time. This screening test assesses coagulation activation via the extrinsic and common pathways. The *thrombin time* (TT) measures the time for clot formation to occur in citrated plasma after addition of thrombin. It reflects the amount and quality of fibrinogen and the rate of fibrin formation. The reasons for abnormal aPTT, PT, and TT values and their clinical relevance are shown in Table 17-4.

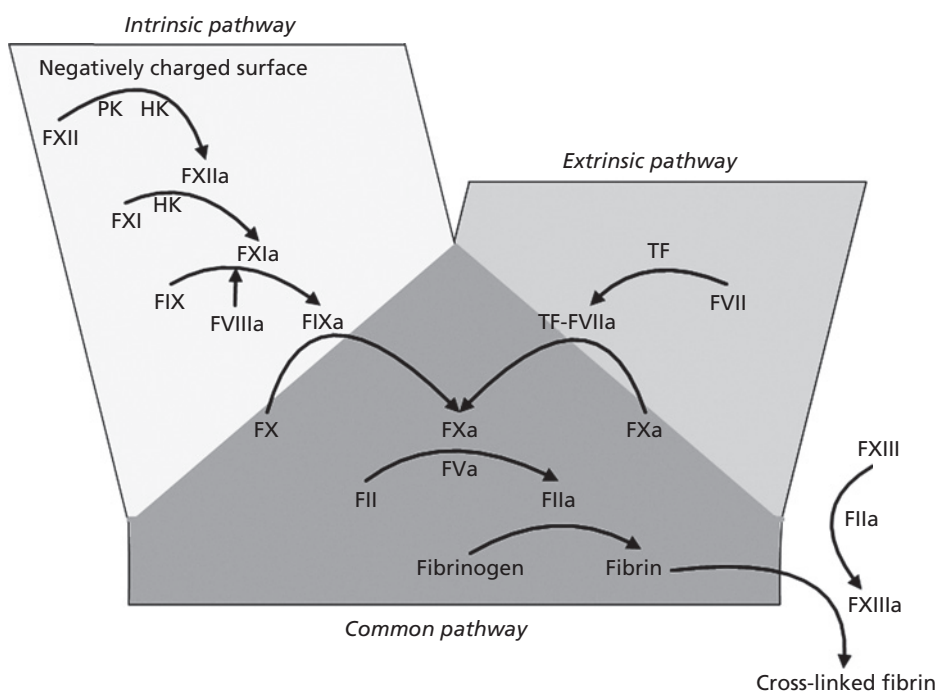


**Figure 17-4.** Classical model of the coagulation cascade, with intrinsic, extrinsic, and common pathways. F = factor; HK = high-molecular-weight kininogen; PK = prekallikrein; TF = tissue factor.

**Table 17-4.** Reasons for an Abnormal Activated Partial Thromboplastin Time, Prothrombin Time, and Thrombin Time

| Abnormal aPTT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | Prolonged PT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | Prolonged TT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Associated with hemorrhage, prolonged</b> <ul style="list-style-type: none"> <li>Fibrinogen, prothrombin, Factor V, X, VIII, IX, or XI deficiency</li> <li>Vitamin K deficiency or antagonism by oral anticoagulants or beta-lactam antibiotics</li> <li>Specific clotting factor inhibitors</li> <li>Unfractionated heparin and low-molecular-weight heparins</li> <li>Thrombin inhibitors, eg, hirudins, argatroban</li> </ul> <b>No association with hemorrhage or thrombosis, prolonged</b> <ul style="list-style-type: none"> <li>Prekallikrein or high-molecular-weight kininogen deficiency, severe Factor XII deficiency</li> <li>Transient phospholipid antibodies, predominantly in children with infections</li> <li>Increased hematocrit</li> <li>Heparin contamination</li> <li>Partially activated sample</li> </ul> <b>Associated with thrombosis</b> <p><i>Prolonged:</i></p> <ul style="list-style-type: none"> <li>Lupus anticoagulant</li> <li>Moderate Factor XII deficiency?</li> </ul> <p><i>Shortened:</i></p> <ul style="list-style-type: none"> <li>Increased plasma levels of fibrinogen, Factor VIII, or Factor IX; coagulation activation</li> </ul> | <b>Associated with hemorrhage</b> <ul style="list-style-type: none"> <li>Fibrinogen, prothrombin, Factor V, X, or VII deficiency</li> <li>Specific clotting factor inhibitors</li> <li>Vitamin K deficiency or antagonism by oral anticoagulants or beta-lactam antibiotics</li> <li>High doses of unfractionated heparin</li> <li>Thrombin inhibitors, eg, hirudins, argatroban</li> <li>Inhibition of fibrin polymerization: fibrin(ogen) degradation products, monoclonal gammopathies, dysfibrinogenemia</li> </ul> <b>No association with hemorrhage</b> <ul style="list-style-type: none"> <li>Increased hematocrit</li> <li>Inhibition of fibrin polymerization: dysfibrinogenemia</li> </ul> <b>Associated with thrombosis</b> <ul style="list-style-type: none"> <li>Lupus anticoagulant</li> <li>Inhibition of fibrin polymerization: dysfibrinogenemia</li> </ul> | <b>Associated with hemorrhage</b> <ul style="list-style-type: none"> <li>Heparin</li> <li>Inhibition of fibrin polymerization: fibrin(ogen) degradation products, monoclonal gammopathies, dysfibrinogenemia</li> <li>Hypofibrinogenemia</li> </ul> <b>No association with hemorrhage or associated with thrombosis</b> <ul style="list-style-type: none"> <li>Inhibition of fibrin polymerization: dysfibrinogenemia</li> </ul> <b>Associated with thrombosis</b> <ul style="list-style-type: none"> <li>Inhibition of fibrin polymerization: dysfibrinogenemia</li> </ul> |

Hereditary dysfibrinogenemia may be associated with bleeding or thrombosis or both, or not associated with any symptoms. aPTT = activated partial thromboplastin time; PT = prothrombin time; TT = thrombin time.

The main use of aPTT is for screening coagulation defects and inhibitors. The test is relatively sensitive to Factor VIII, IX, XI, and XII deficiencies and to prekallikrein and HMW kininogen deficiencies. It may also be prolonged by more severe defects of fibrinogen, Factors V and X, and fibrin polymerization. Factors affecting the clotting response of the aPTT test include the composition and concentration of phospholipids, the type of contact activator and buffer, and the length of incubation with the plasma. An aPTT should be sufficiently sensitive to record single clotting factor deficiencies of 30% of normal or less.<sup>88</sup> Some aPTT reagents fail to detect individual coagulation factor deficiencies as low as 20%. When used as a screening test for lupus anticoagulant, the detection sensitivity is predominantly determined by the type and concentration of the phospholipid reagent. The aPTT is still the most widely used method for monitoring treatment with unfractionated heparin. Conditions associated with a prolonged aPTT but not with any increased risk of bleeding include Factor XII, prekallikrein, and HMW kininogen deficiencies; lupus anticoagulants; transient phospholipid antibodies; increased hematocrit resulting in citrate anticoagulant excess; and heparin contamination of samples. Recent findings suggest that moderate Factor XII deficiency might be associated with vascular mortality.<sup>89</sup> Transient phospholipid antibodies and

concomitant prolonged aPTT without any clinical symptoms are frequently observed in children suffering from repeated infections.<sup>90</sup> Increased plasma levels of fibrinogen, Factor VIII, and Factor IX sometimes cause an abnormally short aPTT, which has been associated with overall poor prognosis and venous thromboembolism.<sup>91,92</sup>

The PT reflects changes in the three vitamin-K-dependent clotting factors (prothrombin, Factors VII and X) and the non-vitamin-K-dependent Factor V. Its sensitivity to clotting factor deficiencies or to vitamin K deficiency or antagonism by oral anticoagulants strongly depends on the source and type of TF thromboplastin reagent. PT prolongations not associated with clotting factor deficiencies may be caused by lupus anticoagulants, increased hematocrit, or impaired fibrin polymerization. Because the heparin sensitivity of PT reagents is reduced by adding heparin neutralizing substances, only very high unfractionated heparin concentrations may cause prolonged PT. The PT is still the most widely used test for monitoring treatment with warfarin or other oral anticoagulants. For this purpose, the PT is calibrated against the international reference thromboplastin and given as international normalized ratio (INR).<sup>93</sup> Therapeutic thrombin inhibitors such as hirudins or argatroban also prolong PT substantially.



The TT detects an impaired fibrin polymerization resulting from fibrin(ogen) degradation products, monoclonal gammopathies, or dysfibrinogenemias more sensitively than the aPTT and PT. Hereditary dysfibrinogenemia is heterogeneous and may be associated with bleeding and/or thrombosis, or may be asymptomatic.<sup>28</sup> Unfractionated heparin causes a marked prolongation of the TT. The *reptilase time* performed by adding the snake venom reptilase to citrated plasma is unaffected by heparin and even more sensitive to impaired fibrin polymerization than is the TT. Because none of the coagulation screening tests are usually prolonged until the fibrinogen level falls below 0.1 g/L, a specific assay for *clottable fibrinogen* should be included in the basic screening tests for detecting coagulation factor deficiencies. Functional fibrinogen is either measured by the Clauss' method on the basis of thrombin-induced clotting or calculated from the change of turbidity during measurement of the PT (PT-derived fibrinogen).

Coagulation screening tests do not detect Factor XIII deficiency, which must be confirmed by appropriate assays.

There is presently no simple and adequately sensitive screening test for detecting mild *hyperfibrinolysis*. D-dimer latex agglutination and turbidimetric and enzyme-linked immunosorbent assays quantify the plasmin-degraded products of cross-linked fibrin in plasma using monoclonal antibodies. However, elevated D-dimer levels are found in numerous clinical conditions that are not inevitably associated with hyperfibrinolysis-induced bleeding. Severe hyperfibrinolysis can be diagnosed easily by markedly elevated D-dimers, prolonged TT and reptilase time, and low fibrinogen, plasminogen, and plasmin inhibitor levels. However, the detection of mild hyperfibrinolytic states associated with bleeding continues to be a diagnostic challenge.

The need for "global screening tests" allowing on-site assessment of the overall hemostatic potential has resulted in further developments of old assays using new technologies. Thromboelastography originally described by Hartert<sup>94</sup> is able to detect thrombocytopenia, platelet dysfunction, coagulopathies, impaired fibrin stabilization, and hyperfibrinolysis when performed in whole blood.<sup>95</sup> Although the test has been used successfully in guiding blood component administration in hepatic and cardiac surgery,<sup>96-98</sup> validation studies are lacking on its sensitivity and specificity to detect mild disorders of hemostasis in all clinical settings. Small studies demonstrated a low sensitivity of thromboelastography to detect platelet dysfunction.<sup>99,100</sup> The endogenous thrombin potential as measured by a thrombin generation curve invented by Hemker<sup>101</sup> may be a useful tool for estimating both the risk of bleeding and thrombosis. However, data justifying its use in clinical routine are still lacking.

## Summary

Plasma is used therapeutically or as starting material for manufacturing albumin, immunoglobulins, protease inhibitor, clotting factor, and coagulation inhibitor concentrates. Plasma protein

levels are subject to wide intra-individual and interindividual variation. Age, gender, ABO blood group, acute phase reactions, physical exercise, mental stress, and hormones substantially influence the plasma levels of therapeutically used plasma proteins. Hemostasis is now understood to involve complex interactions between vessel walls, platelets, blood cells, proteins circulating in blood or being fixed to cells, and other humoral factors such as ionized calcium. Clinically useful coagulation screening tests include activated partial thromboplastin time, prothrombin time, and thrombin time. The detection of mild hyperfibrinolysis and of mild coagulation disorders requires additional specific assays, a clinical history, and an examination.

## Disclaimer

The author has disclosed no conflicts of interest.

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# 18 Immunoglobulins

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With worldwide use exceeding 60 tons/year, immunoglobulin (Ig) preparations for prophylactic and therapeutic use prepared from pooled donor plasma represent a significant plasma derivative. Because pool sizes may involve over 10,000 donors, the plasma from a given donor will reach a large number of recipients. The worldwide yearly volume of plasma processed in approximately 70 fractionation centers amounts to about  $20 \times 10^6$  L—and is increasing.<sup>1</sup> That volume is now driven by the need for intravenous and subcutaneous preparations, replacing the former drivers, which were albumin and Factor VIII. Efficacy and safety records, including hemovigilance tracking, are favorable and treatment with intravenous immune globulin (IVIG) and subcutaneous immune globulin (SCIG) preparations are possible in the outpatient and home-care settings. Understanding the mechanism of action for some indications has advanced the clinical rationale for utilization. Off-label use outnumbers labeled indications and continues to expand. Preparations are also being used with increased frequency in developing countries.<sup>2</sup> Treatment with Ig preparations attempts to replace antibodies in immune deficiencies or modulate the immune system in autoimmune disorders. In the latter, Ig therapy can result in a decrease in corticosteroid use. Ig therapy may be enhanced with hyperimmunoglobulins and monoclonal antibody (MoAb) therapy. Immunoglobulins appear throughout the body, are abundant in the bloodstream, and represent about one-fourth of total plasma protein concentration. They are synthesized by B lymphocytes and plasma cells with a synthesis rate as high as 2000 molecules per second per single B lymphocyte. The pooling of plasma from large numbers of donors results in a product that contains antibodies directed toward a wide range of antigens. The production of Ig preparations is a complex and costly process that takes 6 to 8 months from the time plasma is collected until the product is available for use.

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### Immunologic Background for Immunoglobulin Therapy

The function of immunoglobulins is reflected in their structure, with the recognition site for antigens found at the outer tips of two branches. The effector part (“business end”) is endowed with the capacity to activate complement and to bind to Fc receptors. Once bound to a cognate antigen at the recognition Fab site, the resulting complex expresses different functions depending principally on its size and solubility.<sup>3</sup> Antibodies can fix the antigen to lymphoid tissues for the prolonged periods required for antigen recognition, using at least in part, the Fc gamma receptor IIB,<sup>4</sup> or they can present the antigen to monocyte-macrophages in the reticuloendothelial system for removal and disposal. The paired polypeptide chains, two heavy (H) chains and two light (L) chains, each contain a constant region shared by different clones and a variable (V) region (ie, a region that differs from one antibody specificity to the next). In contrast to the constant region genes, almost every B cell expresses a different pair of heavy- and light-chain variable region genes, a diversity that is generated during B-cell development by somatic recombination at the DNA level of three major building blocks:  $V_H$  (variable), D (diversity), and  $J_H$  (joining) segments. Each block has several genes that recombine and create diversity, enhanced by imprecise joining and the incorporation of untemplated nucleotides where the genes join (junctional diversity). The affinity of the antigen-antibody interaction is subsequently improved by somatic hypermutation of the rearranged genes followed by further rounds of selection, a process known as affinity maturation.

The repertoire of antibodies in a donor's plasma have specificity that is passed on in Ig preparations, resulting in availability for the recipient of therapeutic and prophylactic antibodies with high efficiency. The targeting of antibodies to specific antigens has been discovered in the example of infectious antigens distributed on pathogenic microorganisms, such as bacteria and viruses. A sufficient antibody response has to be present in the host in order for that host to remain disease-free. Beyond defense, the tasks of antibodies are part of a network that, in collaboration

with cellular immunology, participates in the homeostasis of the organism, including internal regulation of the immune system, apoptosis (Fas antibodies, see below<sup>5</sup>), and defense against malignant tumors.

Once antigenic stimulus ceases, healthy individuals develop a memory state and maintain low-grade antibody production. When reexposed to the same antigen, the healthy individual increases production of antibody. It remains elusive why certain self-proteins induce an autoimmune response.<sup>6</sup> One immunologic hypothesis is that only modified or altered self-proteins may become autoimmunogenic. Such alterations could reflect genetic polymorphisms revealed by analysis of amino acid sequence variability of known human autoantigens. Autoantigens have been found containing significantly more single nucleotide polymorphisms (SNPs) than other human genes.<sup>6</sup> Such findings might offer an explanation for autoimmune responses through allogeneic exposure. Besides other contributing factors in autoimmunity, SNPs may represent an essential prerequisite for the primary induction of an autoimmune response.<sup>7</sup> Memory and low-grade antibody production thus includes the response to autoantigens, eg, thyroglobulin or deoxyribonucleic acid, which results in production of normal autoantibodies.<sup>8</sup>

Excessive autoantibody synthesis is controlled by anti-idiotypic suppression. By virtue of their specific V regions, autoantibodies express an antigen-specific reactive site termed an *idiotype*. This site, in turn, can elicit the formation of a second antibody (Ab2), termed an *anti-idiotypic*. Anti-idiotypes can be antigen-site reactive, binding to the very cleft between the H and L chains of the outer tip of the Fab fragment where antigen is bound. Alternatively, anti-idiotypes can be frame-reactive and bind to outer parts of the Fab fragment and leave antigen binding by the idiotype-carrying primary antibody (Ab1) unaffected (Fig 18-1). Those anti-idiotypic antibodies that bind to the distal tip of Fab arms (ie, to the complementary determining region, or hypervariable region) are less likely to produce bivalently associated ringed dimers<sup>9</sup> than antibodies that bind to more proximal epitopes, projecting laterally from the surface of the Fab arms. Ab2 reacts with Ab1 not only in the fluid phase but also when Ab1 is expressed as surface immunoglobulin on B lymphocytes. In these cases, Ab2 may compromise synthesis of Ab1 and perhaps switch off the B-lymphocyte clone that produces Ab1.

These steps of the immune response (formation of specific Ab1 antibodies followed by Ab2 anti-idiotypes) explain why the design of drugs to replace these antibodies or the use of MoAbs remains complex at present.<sup>10</sup>

The therapeutic potential of IVIG remains to be elucidated in the field of influencing cellular signal transduction pathways involved in inflammation and immunity.<sup>11</sup> The goal of signal transduction is the alteration of gene expression to allow the cell to respond to the environmental signals. There are five fundamental components to every signal transduction pathway, which cells use to modulate immediate and long-term changes in gene expression. They are: 1) the ligand, 2) the receptor, 3) the cytoplasmic transduction molecule(s), 4) the transcription factor(s), and 5) the negative regulator(s). The presence in IVIG of antibodies against

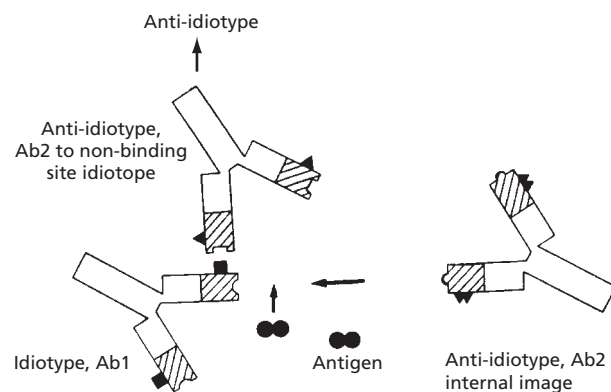


**Figure 18-1.** The prefix “idio” comes from Greek language and means private. Thus, every antibody specificity directed against a given antigen, because of the specific recognition of this antigen, carries its own particular structural element at the site of the hypervariable regions termed idiotype. The immune system recognizes this idiotype and readily makes antibodies against it, termed anti-idiotypic. The latter may bind to the former and thus form idiotype-anti-idiotypic complexes. Such complexes are important in homeostasis of the immune system as a whole, because they occur not only in fluid phase but also on cell surfaces carrying Fc receptors or surface immunoglobulins or both. The idiotype-anti-idiotypic network is further regulated by polyspecific polyclonal IVIG preparations, which also carry members of this network present in the normal plasma donor population, which supplied starting material for their preparation.

one or several of such components<sup>12</sup> is now obvious. Overactivation or defective inhibition of selective receptors, kinases, and transcription factors can convert a normal immune response into a chronic disease state. Decades ago, researchers were reluctant to study mechanisms of action of human IVIG in animal models because of concern about heterologous immune responses. However, this concern has waned in the setting of short-term experiments that do not allow heterologous immune responses to become active.<sup>13</sup> Targets for intervention have been identified and the promise of rational drug design and/or IVIG application has come to fruition. An example is caspases (cysteine proteases), which play essential roles in apoptosis and inflammation. Once caspase-3 is processed, apoptosis is irreversible, because this enzyme directly activates the DNases, lipases, and proteases that destroy the cell. These cysteine proteases use a cysteine residue to cut the proteins, and are called caspases because the cysteine residue cleaves the substrate proteins at the aspartic acid residue. On the basis of the discovery a decade ago that IVIG contains antibodies against Fas,<sup>14</sup> researchers theorized that part of the antiinflammatory effect of IVIG is the result of its capacity to induce caspase-dependent cell death in Fas-sensitive cells. The Fas ligand or FasL is a type II transmembrane protein that belongs to the tumor necrosis factor (TNF) family. Fas ligand binds with its receptor to induce apoptosis. Fas ligand-receptor interactions play an important role in the regulation of the immune system. Induction of caspases upon Fas-ligand binding (CD95) is essential in cells for apoptosis, one of the main types of programmed cell death in developmental and most other stages of adult life, because caspases are “executioner” proteins in the cell.

**Table 18-1.** Comparison of Selected Intravenous Immunglobulin Preparations

|                     | Flebogamma                    | Gammagard                 | Polygam                                | Octagam/<br>Newgam                | Carimune                           | Tegelin                   |
|---------------------|-------------------------------|---------------------------|----------------------------------------|-----------------------------------|------------------------------------|---------------------------|
| Distributor         | Grifols<br>(Barcelona, Spain) | Baxter<br>(Deerfield, IL) | American Red Cross<br>(Washington, DC) | Octapharma<br>(Vienna, Austria)   | CSL Behring<br>(Bern, Switzerland) | LFB (Les<br>Ulis, France) |
| mOsm/L              | 240-350                       | 363 (5%)<br>1250 (10%)    | 363 (5%)<br>1250 (10%)                 | 30-380 (Octagam)<br>1250 (Newgam) | 444-1020<br>5% D-1020              | 350-400                   |
| Sugar               | Sorbitol                      | Glucose                   | Glucose                                | Maltose                           | Sucrose                            | Saccharose                |
| Formulation         | Liquid                        | Lyophilized               | Lyophilized                            | Liquid                            | Lyophilized                        | Lyophilized               |
| Storage temperature | RT                            | 2-6°C                     | RT                                     | RT                                | RT                                 | RT                        |
| Viral depletion     | Pasteurization + S/D          | S/D                       | Cold ethanol                           | S/D                               | pH 4; nanofiltration               | pH 5.2; ethanol           |
| Size (grams)        | 0.5/2.5/5/10/20               | 2.5/5/10                  | 1/3/6/12                               | 1/2.5/5/10/20                     | 3/6/12                             | 0.5/2.5/5                 |

RT = room temperature; S/D = solvent/detergent-treated.

Some caspases are also required in the immune system for the maturation of cytokines. Failure of apoptosis is one of the main contributions to tumor development and autoimmune diseases. This, coupled with the unwanted apoptosis that occurs with ischemia or Alzheimer's disease, has enhanced the interest in caspases as potential therapeutic targets. Concurrently, the CD33-related sialic-acid-binding Ig-like lectins (Siglecs) are cell surface receptors and members of the immunoglobulin superfamily (IgSF). Siglecs recognize sugars that are expressed on most immune cells, where they exert inhibitory signaling functions and downregulate cellular activation pathways via cytosolic immunoreceptor tyrosine-based inhibitory motifs. Human T cells are striking exceptions and do not express these molecules. The human-specific loss of T-cell Siglec might explain the hyperactivity of helper cells in AIDS and chronic active hepatitis.<sup>15</sup> IVIG activates Siglec 9, the receptor on the surface of neutrophils, thus inducing neutrophil cell death. One group of investigators studying the action mechanisms of IVIG concludes from these observations that IVIG exerts its immunoregulatory effects under pathologic conditions, at least in part, through its impact on Siglecs.<sup>16</sup>

With the advent of MoAbs, clinicians now have at their disposal an efficient antibody preparation devoid of unnecessary ballast (ie, antibodies carrying specificities not necessary for the recipient). In recent years, antibodies of defined specificities have also been engineered using gene rearrangement technology through phages, eliminating the immunization of any laboratory animals. Gene clusters encoding single-chain Fv (scFv) fragments can be made by randomly combining H- and L-chain V genes using the polymerase chain reaction (PCR), and the combinatorial library (>10<sup>7</sup> members) can be cloned for display on the surface of a phage. A single large phage display library can be used to isolate human antibodies against any antigen, bypassing both hybridoma technology and immunization. But even with the advent of engineered humanized antibodies or tailor-made antibodies with single or dual specificities (diabodies<sup>17</sup>), a number of difficulties have been encountered during administration, including production of

inhibitory anti-idiotypes, again suggesting that the complete network conferred by Ig preparations is difficult to replace with monospecific therapy.

### Polyspecific Immune Globulin Preparations for Prophylaxis and Therapy

IVIG, SCIG, and intramuscular Ig (IMIG) preparations are biologicals and stable blood derivatives. The Food and Drug Administration (FDA) uses the term immune globulin. In France, they are called MDS (médicaments dérivés du sang, or blood-derived drugs). Stringent requirements for IVIG have been set by the World Health Organization (WHO) and the European Committee for Proprietary Medicinal Products (CPMP; <http://eudraportal.eudra.org/>) (Table 18-1).

Immunoglobulins are hydrophobic and tend to adhere to surfaces during purification. Aggregated IgG molecules are common in purified IMIG and SCIG preparations and make up 10% to 20% of the total protein. Dimer formation is reversible,<sup>3</sup> but higher order polymers are stable and will cause serious side effects if infused intravenously. The preparation method of IVIG or SCIG is developed to maintain biological activity, provide a high degree of pathogen safety, and produce high yields of highly purified immunoglobulins.

More than 90% of total protein should be monomeric IgG with intact Fab and Fc functions. The subclasses 1 through 4 of IgG should be present at the same ratio as found in normal plasma. The half-life after infusion should be the same as the half-life of normal IgG. No complement-activating IgG polymers should be present.

Whenever Ig products are in limited supply, it is important to develop protocols that will ensure that patients in greatest need receive their treatments. Some countries, such as Australia, have increased the postmarketing supervision of Ig therapy.<sup>18</sup> The worldwide shortage<sup>19</sup> makes it necessary to review the appropriateness of Ig therapy for certain disorders.

Indeed, there is no acceptable alternative therapy to immunoglobulin in patients with primary immunodeficiency diseases (PID), where IVIG or SCIG is the treatment of choice. Gene therapy using allogeneic hematopoietic stem cell transplantation is necessary in certain patients such as those with severe combined immune deficiency (SCID) or other PIDs where Ig therapy and/or other therapies are ineffective. Gene therapy remains dependent on availability of HLA-matched sibling donors and gene-modified autologous marrow transplantation is not established.<sup>20</sup> Inclusion of IVIG in the WHO Model List of Essential Medicines will facilitate the possibilities of diagnosis and treatment of immunodeficient patients in developing countries. In addition, its inclusion will contribute to improving the access of developing countries to a range of blood-derived medicinal products already on the Model List that are used for replacement therapy in a variety of life-threatening diseases. For that reason, these patients are among those who should receive the highest priority in the use of IVIG and SCIG. Progress in simple IgG measurement using quantitative estimations of IgG subclasses by turbidimetric, nephelometric, or radial immunodiffusion technology has resulted in more precise dosages for patients. The use of IVIG or SCIG is guided by IgG concentration measurements before and after injection in order to study depleted levels (“trough levels”) and therapeutic IgG increments (Fig 18-2). Such measurements are important for pharmacoeconomic reasons and to ensure the appropriate dosing.<sup>21</sup>

Therefore, each patient must receive individualized therapy in order to optimize the response. Beneficial and cost-effective therapy results when the minimal effective dosage is used. PID patients who depend on lifelong IgG replacement therapy require meticulous laboratory follow-up<sup>22</sup> ([www.scid.net](http://www.scid.net)). Generally, health-care providers pay insufficient attention to laboratory expertise in the diagnosis and follow-up of immunodeficiency.

The clinical pathology laboratory performs the following functions:

- Ascertain the diagnosis, which leads physicians to prescribe IgG replacement.
- Monitor long-term safety and efficacy of IgG replacement.
- Ensure on-site good clinical practice for IVIG or SCIG administration using selected point-of-care tests.<sup>23</sup>

### Assistance of Laboratory Procedures in Prescribing Immunoglobulins

Because the main laboratory test for patients receiving Ig replacement therapy is quantitation of IgG levels, it is assumed that in published studies these tests are performed under standards for good laboratory practice. Agencies such as the National Academy of Clinical Chemistry, the International Federation of Clinical Chemistry and Laboratory Medicine, or the European Autoimmunity Standardization Initiative Group supervise standardization, traceability, and reproducibility. The preferred technique to quantitate immunoglobulins in fluid phase is nephelometry.

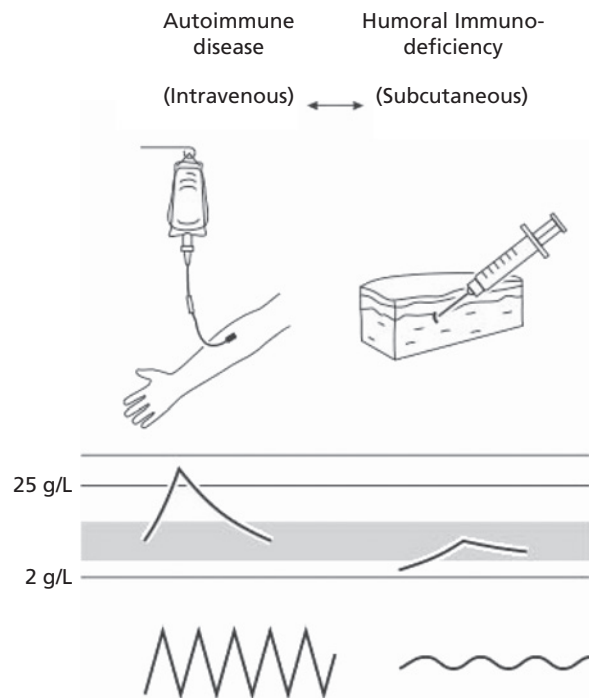


**Figure 18-2.** Two of the major injection sites for immunoglobulin preparations are depicted. The pharmacokinetic profile of repeat administrations is also given.

The plasma fractionation industry has developed production procedures for highly concentrated IgG preparations. Some IVIG preparations can reach 10% or 12% levels and those for SCIG administration can reach 15% to 17%, which raises concerns about hyperviscosity.<sup>24</sup> A final viscosity of  $>10$  centipoise for SCIG preparations increases the probability of local reactions. The viscosity of the solution may be the cause of the local site reactions that patients receiving SCIG experience. Viscosity techniques have now reached high measurement accuracy, provided the temperature of the fluid is adjusted. Close laboratory monitoring of IVIG and SCIG therapy will support appropriate dosing of a product that is often in short supply.

### Primary Immunodeficiency States Requiring Immunoglobulin Substitution

The normal average range of total IgG in humans is between 6 and 16 g/L, so that some healthy individuals are well protected against infection with approximately 7 g/L, whereas others require about 14 g/L to be free of infections. There are no gender or age differences and the levels of many antibody specificities (except those induced by vaccination) remain stable over time.<sup>25,26</sup> Clinical immunodeficiency can result from a deficiency of a single IgG subclass in the presence of normal, or elevated, levels of other IgG subclasses with non-functional antibodies. A typical example for such a disease



state is the specific polysaccharide antibody deficiency syndrome.<sup>27</sup> Therefore, assessment of both the concentration and the functionality of the specific antibodies where the clinical evidence dictates is superior to the measurement of only the total IgG.<sup>28</sup> More than three infectious episodes in a year that are resistant to antibiotic treatment over 4 weeks, make measurement of IgG along with basic complement system assays mandatory. Abnormal results in such screening indicate that a differential diagnosis can be made, including common variable immunodeficiency<sup>29</sup>; Wiskott-Aldrich syndrome; SCID; transient hypogammaglobulinemia of infancy; immune dysregulation, polyendocrinopathy, enteropathy, x-linked (IPEX) syndrome; chronic granulomatous disease; ataxia-telangiectasia; complement deficiencies; or mitochondrial depletion syndrome with T-cell immunodeficiency.<sup>30</sup> IgG subclass measurement is not advisable if the hypogammaglobulinemia is  $<2\text{ g/L}$ . However, with a moderate deficiency of  $<5\text{ g/L}$  IgG, a selective subclass deficiency must be excluded.

The hypogammaglobulinemia spectrum opens up a large array of further associations, such as metabolic disorders, deficiency of transcobalamine, and an increasing number of possible chromosomal abnormalities, all of which might be classified under the heading of primary forms of humoral immunodeficiency. A complete patient and family history improves diagnostic tracking before comprehensive laboratory testing. Toxic drug reactions with antimalarial agents, captopril, carbamazepine, or gold compounds may be followed by hypogammaglobulinemia. Malignant disease, typically chronic lymphatic leukemia and non-Hodgkin lymphoma, should be excluded as well. If malignant disease is suspected, marrow examination, colonoscopy, and thorax x-ray are performed on a case-by-case basis. Hypogammaglobulinemia is a laboratory finding in myotonic dystrophy with accelerated catabolism of IgG and in protein-losing enteropathy with intestinal lymphangiectasis. Some types of clinically overt immunodeficiency states with normal IgG levels require assessment of the complement system. Selective deficiencies of C1q, C4, or C2 suggest a genetic background, whereas C5-9 deficiencies are associated with meningococcal infection. Deficiency of C3 is also possible but the clinical evidence manifests in early infancy with *Hemophilus influenzae* and pneumococcal infections being associated with renal insufficiency. Preconditioned complement analysis kits are available for this purpose.

## Use of Immunoglobulin Therapy in Secondary Immunodeficiencies

IVIg therapy is useful in treating hypogammaglobulinemia induced by chemotherapy regimens, especially with intensified CHOP regimens and/or when associated with lymphoid irradiation.<sup>31</sup> Standard immunosuppressive therapy of autoimmune diseases with alkylating agents or with mycophenolic acid<sup>32</sup> might also, in some patients, require the use of IVIG.

However, this can be minimized by optimal tailoring of specific immunosuppressive drug therapy adapted to each patient's genomic makeup, a domain referred to as pharmacogenomics.<sup>33</sup> To determine the reason for hypogammaglobulinemia in its patient population, a large tertiary care pediatric hospital recently completed a review of all cases of laboratory-defined hypogammaglobulinemia between August 1990 and June 2006.<sup>34</sup> IgG levels in 8304 samples were analyzed and 1295 specimens from 680 patients exhibited hypogammaglobulinemia. In addition, 172 patients with an immune deficiency were identified; 7% of these had SCID. Evaluation of all patients with IgG levels less than half of the lower limit identified 122 generally immunodeficient patients, 33% of whom had primary immune deficiency. This study provides a framework for considering causes of hypogammaglobulinemia. At the study institution, hypogammaglobulinemia was found most often as a secondary immune deficiency caused by chemotherapy or complex cardiac anomalies.<sup>34</sup> Thus, IVIG and SCIG will most certainly be used in treating secondary immunodeficiencies. MoAbs that have been employed in cancer treatment have also demonstrated efficacy in nonmalignant diseases. Rituximab, a CD20 humanized MoAb, may induce hypogammaglobulinemia by virtue of its B-cell targeting but also other MoAbs, such as alemtuzumab (anti-CD52), express considerable infectious toxicity.<sup>35</sup> As more side effects of rituximab therapy are discovered<sup>36</sup> and the mechanism of side effect induction is elucidated, it may be determined that some of these side effects can be effectively managed with IVIG therapy.

## Intravenous Immune Globulin in Infectious Diseases

Immunoglobulins with antigen specificity that confers specific antibody reactivity to previously nonimmune individuals can be used to treat infectious diseases. Schedules, dosages, and routes of administration of immunoglobulins differ. Physicians should be familiar with the available IMiG preparations used in prophylaxis of infectious diseases as follows:

- Standard immunoglobulin preparations
  - hepatitis C
  - rubella
  - rubeola
- Hyperimmunoglobulins (higher specific antibody content)
  - hepatitis A
  - hepatitis B
  - diphtheria
  - rabies
  - rhesus isoimmunization
  - tetanus
  - tick encephalitis virus
  - varicella zoster

The IMiG and SCiG preparations may be sufficient for pre-exposure prophylaxis, but to prevent an outbreak in exposed

individuals an IVIG preparation may be needed. For anti-infectious immunoglobulins, donors with high titers are a source for antibody-enriched plasma. It can be difficult to determine the appropriate dosing with hyperimmune Ig preparations when treating complex antigens, because the preparation may not contain the necessary specificity of the antigen in question.

In diseases with hypogammaglobulinemia in which specific antibodies are insufficiently synthesized, resulting in more than three bacterial respiratory, intestinal, or urinary tract infections per year, IVIG infusions or SCIG preparations<sup>22,37,38</sup> must be administered on a regular basis. SCIG preparations, using liquid pasteurized polyvalent human 16% concentration (160 mg/mL) are appropriate for certain patients. Those patients who prefer to be treated at home or who cannot tolerate IVIG products are appropriate candidates. Patients can be trained and supervised once per week at a local hospital for four to six training sessions and tested for their practical skill and confidence in the SCIG administration route before being transferred to self-injections at home; parents give injections to children or supervise the treatment.<sup>39</sup>

It is well known that most infections provoke hypogammaglobulinemia except rubella, cytomegalovirus (CMV), and mycoplasma, which cause secondary hypogammaglobulinemia. In such cases, the laboratory will determine the titers of a selection of viral antibodies because anti-bacterial titers will not be informative. Clinically overt infections caused by some viruses might occur as an inherited monogenic trait. PID is not restricted to children<sup>40-42</sup> and is sometimes diagnosed in adults with recurrent infections and low IgG levels. The ongoing improvement of immunologic diagnostic procedures (using microarray technologies, genomic, and proteomic approaches), as well as recent discoveries in basic and clinical immunology, will likely increase identification of patients for the treatment.

## Intravenous Immune Globulin in Immune-Mediated Disease

A special feature of alloimmune and autoimmune diseases is the potential to affect virtually any system or organ of the body. Systemic autoimmune diseases are characterized by the production of autoantibodies that recognize a diverse array of cytoplasmic and nuclear antigens. Therefore, it is important to distinguish between the terms “autoimmunity” and “autoimmune disease.” The former is an adaptive immune response (T- or B-cell mediated) against self-antigens either with or without concomitant clinical manifestations. Autoimmune disease implies the existence of adverse manifestations, such as kidney disease, arthritis, rashes, and pleuritis arising as a consequence of a T- or B-cell mediated response to self.<sup>43</sup> The immune system must therefore discriminate between self and non-self. When self/non-self discrimination fails, the immune system destroys cells and tissues of the body and as a result causes autoimmune diseases. Immunologists are gaining insight into the body’s capacity to prevent autoimmune disease by maintaining

autoimmune recognition: regulatory T cells actively suppress activation of the immune system and prevent pathologic self-reactivity, ie, autoimmune disease. The critical role regulatory T cells play within the immune system is evidenced by the severe autoimmune syndrome that results from a genetic deficiency in regulatory T cells. As insight into the cellular network responsible to tune autoimmune recognition improves, the understanding of IVIG action mechanisms becomes possible in more detail.<sup>44</sup> If IVIG is efficacious in such different diseases as neurologic, hematologic, endocrinologic, and other disorders, there may be a common denominator among mechanisms. Accepted indications for IVIG in treating immune-mediated disease are the following:

1. Replacement in primary and secondary immunodeficiency diseases (such as in B-cell lymphoproliferative disorder).
2. Allogeneic marrow transplantation.
3. Kawasaki disease.
4. Guillain-Barré syndrome.
5. Chronic inflammatory demyelinating polyneuropathy (CIDP).
6. Myasthenia gravis.
7. Dermatomyositis.

The hematologic indications include immune thrombocytopenic purpura (ITP), pure red cell aplasia, and alloimmune-mediated thrombocytopenias.

Other situations where IVIG is lending increasing support, based on ongoing clinical studies, include the following:

1. Systemic vasculitis.
2. Hemorrhagic diathesis caused by Factor VIII inhibitor.
3. Recurrent pregnancy loss.
4. Prevention of neonatal sepsis.
5. Infections in polytraumatized and postsurgical patients.
6. Solid organ posttransplantation immunomodulation.

For most of these indications, observations of single cases favorably responding to IVIG were followed by the classic sequence of the four phases of clinical studies.

Many years of study on immune-complex-mediated diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis, and ankylosing spondylitis further define patients who will or will not respond to IVIG therapy. Thrombocytopenia and secondary myelofibrosis and/or cerebritis accompanying SLE seem to respond, and animal experiments of abrogation of experimental SLE with IVIG show benefit, suggesting there may be a role for IVIG in these diseases. With more in-depth definition of pathophysiologic processes in diseases now closer defined by molecular biologic approaches, MoAbs are becoming targeted tools that will be supported by simultaneous IVIG application.<sup>45</sup>

Conceptual design of a clear mechanism may lead to successful clinical trials of IVIG. Thus, two bleeding patients with autoantibodies against clotting Factor VIII responded to IVIG,<sup>46</sup> because the intrinsic regulation of the antibody network failed and could be reconstituted by IVIG. It appears that the inhibitor-designated suppressors of von Willebrand factor (vWF) cleaving protease ADAMTS13 may in fact be nothing else than autoantibodies. The clinical hemorrhage in the framework of resulting thrombotic thrombocytopenic purpura might be responsive to IVIG,<sup>47</sup> a

condition for which several clinical studies are under way with rituximab and Fresh Frozen Plasma.

As with infectious diseases, the search to help an additional number of autoimmune patients with IVIG therapy might open a new chapter using IgM-enriched preparations, because IgM isotype controls IgG autoreactivity. For the time being, most IgM from fractionated plasma is left unused. Substituting defective IgM with IgM from healthy donors might constitute an additional rationale currently being pursued in clinical studies. An IgM-enriched immunoglobulin preparation is a more potent downregulator of an in-vitro alloantigen-induced proliferation in the mixed lymphocyte reaction than a pure monomeric 7S IVIG.<sup>48</sup> Therefore, some studies now claim that IgM-enriched IVIG might exert a greater therapeutic potential than regular IVIG preparations.

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### **Intravenous Immune Globulin in Neurologic Disorders**

Guillain-Barré syndrome (GBS), myasthenia gravis,<sup>49</sup> dermatomyositis, polymyositis, multifocal motor neuropathy with conduction block, and CIDP have become accepted as labeled indications by numerous health authorities. Furthermore, Lambert-Eaton syndrome and stiff-person syndrome might be additionally responsive to IVIG.<sup>50</sup> There has been substantial progress in explaining the disease mechanisms. It appears that autoantibodies to ganglioside GM antigen on Schwann cells are a major disease-inducing factor, which becomes inhibited by anti-idiotypic antibodies in GBS. IVIG also modulates the levels of proinflammatory cytokines in these diseases, selectively reducing TNF- $\alpha$  and interleukin-1, the levels of which can be correlated with disease severity. In dermatomyositis, IVIG therapy restores intramuscular capillaries by blocking the antibody-dependent formation of C5b-9 and the terminal membrane attack complex of complement that mediates endothelial cell damage. The response of patients with CIDP to IVIG might reflect factors in addition to Fc receptor blockade. In patients with multiple sclerosis (MS), hope exists that IVIG might at least relieve the patients from major symptoms. Several well-controlled clinical trials have been performed in different stages and phenotypes of MS. The results are mixed. Speculations that IVIG might be able to reverse fixed neurologic deficits from MS could not be confirmed. Adding IVIG to the conventional treatment of MS relapses with high-dose intravenous methylprednisolone also did not provide any additional benefits. Similarly, trials failed to establish a role for IVIG in the treatment of secondary or primary progressive MS. Most consistent beneficial results with a reduction of relapse rates and a slowing of disability have been obtained in relapsing-remitting MS including clinically isolated syndromes, although a most recent study did not confirm a reduction of disease activity based on clinical findings and magnetic resonance imaging results.<sup>51</sup> Trial results also suggest that IVIG might serve to suppress an increased recurrence of relapses immediately after delivery. Consequently, IVIG treatment is considered as second-line option for these indications, although there is still uncertainty regarding the actual

mechanism(s) of action and optimal dosage of this treatment.<sup>52,53</sup> Two-hundred and thirty-one patients stratified for primary MS (n = 34) and secondary progressive MS (n = 197) were randomly assigned to receive IVIG 0.4 g/kg/month or placebo for 24 months. Primary endpoints were 1) the time to sustained progression of disease, identified as sustained worsening of the expanded disability status scale (EDSS) score for 3 months, and 2) the improvement of neurologic functions defined by a patient's best EDSS score. Secondary endpoints were the proportion of patients with sustained progression, the relapse rate, the assessment of fine motor skills, visual evoked potentials, contrast sensitivity, depression, and quality of life. Analysis of the intention-to-treat population of combined primary MS and secondary progressive MS patients showed that the mean time to sustained progression was 74 weeks in the IVIG group compared with 62 weeks in the placebo group (p = 0.0406). There was no IVIG-mediated improvement in neurologic functions. More in-depth studies are required to see if IVIG halts progression of MS, at least in combination with MoAbs.<sup>54</sup>

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### **Intravenous Immune Globulin in Cardiovascular Diseases**

The effectiveness of IVIG in Kawasaki disease (a children's illness also known as Kawasaki syndrome or mucocutaneous lymph node syndrome) has encouraged research in other cardiovascular diseases. Kawasaki disease and acute rheumatic fever are the two leading causes of acquired heart disease in children in developed countries. About 80% of the afflicted patients are younger than 5 years of age and males are affected more often than females. Although it is seen in a higher incidence in those of Asian ancestry, it can occur in every racial and ethnic group. More than 1800 cases of Kawasaki disease are diagnosed annually in the United States. In as many as 20% of the children with Kawasaki disease the heart is affected, most commonly the coronary arteries. This can result in aneurysms, thrombosis, and stasis. Other complications include myocarditis, arrhythmias, and valvular dysfunction. IVIG should be given early in the disease course—even in cases in which the heart is not yet affected. If Kawasaki disease has been observed after parvovirus infection, the IVIG may act through neutralizing parvovirus-B19-specific antibodies. However, in most children suffering from this syndrome the etiology remains unknown, and IVIG must be given early in conjunction with aspirin for at least 10 days to prevent the development of coronary artery aneurysms. Dosage should be 1.6 to 2.0 g/kg, administered in divided doses over 2 to 5 days, or 2 g/kg/day as a single dose.

Inflammation of the myocardium is a condition of serious clinical importance with inflammatory etiologies ranging from viral myocarditis, Chagas' disease, and AIDS, and noninfectious causes, including posttransplant cardiac rejection. Initial efforts to abrogate the development of cardiac dilation and dysfunction in patients with inflammatory myocarditis with immunosuppressive therapy were unsuccessful. When children with or without cellular inflammation shown by endomyocardial biopsy were

treated with IVIG, significant improvement was noted in left ventricular function. In a recent study the clinical presentation, severity, and outcome of enteroviral infections in children with malignancy were studied. All cases of enteroviral infections in a University Pediatric Hematology-Oncology Unit were assessed during a 5-year period. Reverse transcriptase PCR, immunohistochemistry, and indirect immunofluorescence assays were performed to document the enteroviral infection and the type of virus.<sup>55</sup> Among 104 patients evaluated for possible enteroviral infection, 55 children had documented enteroviral infection. Severe cardiac manifestations occurred in three of the 55 infected patients (one myocarditis, one dilated cardiomyopathy, one ventricular fibrillation). All patients received supportive care, IVIG, and/or pleconaril.<sup>55</sup> All patients who received high-dose IVIG developed early negative viral load. Infection-related fatality rate was 14.5% (n = 8). Enteroviruses caused more severe and lethal manifestations especially in children with lymphoid malignancy. The administration of high-dose IVIG was beneficial in viremia. Thus, the early therapeutic intervention with high doses of IVIG improves outcome in certain viral diseases. The use of IVIG in virus-induced myocarditis<sup>56</sup> is another area under investigation.

A mechanism of action in these conditions is the capacity of IVIG to deviate deleterious complement activation to innocuous targets with the IgG molecule itself binding C3b. However, the production of inflammatory complement breakdown products that induce vasodilation at the site of inflammation, and the adherence of phagocytes to blood vessel endothelium, support the potential role of MoAbs. Thus, eculizumab or its 25-kD single-chain version (scFv) lacking the whole constant region of the antibody, pexelizumab, may specifically block C5 cleavage<sup>57</sup> and may act similarly to, but more specifically than, IVIG.

## Immunoglobulin Therapy of Dermatologic Diseases

A sizable proportion of the worldwide use of Ig preparations benefits patients suffering from dermatologic disease. Toxic epidermal necrolysis (TEN) and bullous pemphigoid have become clear indications for IVIG therapy<sup>58</sup> and Ig preparations are being used in a number of other dermatologic diseases as well. Severe forms of pemphigus vulgaris were being treated with high-dose corticosteroids and immunosuppressive agents, but the use of IVIG has reduced titers of serum antibodies against keratinocytes, especially where IVIG has been used in conjunction with rituximab.<sup>59</sup> The mechanisms of action of Ig therapy in TEN have been elucidated: IVIG suppresses the proliferation of antigen-specific T cells without inducing apoptosis and the cells remain refractory to induction of apoptosis by CD95 ligation.<sup>14</sup> The infusion of high-dose IVIG is safe, well tolerated, and likely to be effective in improving the survival of patients with TEN. Early treatment with IVIG at a total dose of 3 g/kg over 3 consecutive days (1 g/kg/day for 3 days) is recommended.<sup>58</sup> With the development of several novel biologic immunomodulators, these drugs have

the potential to significantly affect the treatment of some inflammatory conditions in dermatology. For the moment, psoriasis remains the only condition in dermatology for which the use of biologic immunomodulators has been approved by the FDA but an understanding of the mechanisms of action provide an optimistic view for the IgE antagonist omalizumab; the TNF- $\alpha$  antagonists infliximab, etanercept, and adalimumab; and the T-cell response modifiers efalizumab and alefacept.<sup>60</sup>

Although only a minority of dermatologic indications (dermatomyositis, Kawasaki disease) are officially registered<sup>61</sup> and double-blind, placebo-controlled clinical studies are often lacking, the observed efficacy and safety profile of currently available IVIG sometimes makes this a treatment of choice for initiation of therapy or for replacement of more toxic alternatives, such as systemic immunosuppressive medications. With relatively rare diseases it is difficult to perform double-blind studies and therapy is often based on case reports. The increasing use of IVIG has been associated with a further understanding of its mechanism(s) of action, identification of areas of efficacy, and management of side effects. The fact that different brands of IVIG may have different efficacies has been demonstrated in the treatment of Kawasaki disease. Tsai and coworkers<sup>62</sup> studied four different brands of IVIG and determined that differences in efficacy were seen in the four products. Additionally, improvements in the purity, viral safety, and tolerability of IVIG products have been observed in some of the currently available products.<sup>61</sup>

## Intravenous Immune Globulin in Organ Transplantation

Organ transplantation is an important field for IVIG treatment, primarily for patients with high-titered HLA antibodies.<sup>63</sup> In organ transplantation, IVIG prophylaxis for rejection is now considered evidence-based<sup>64</sup> through a number of single case reports.<sup>65</sup> IVIG is now an established therapy for both conditioning of prospectively suboptimal donor/recipient matches and organ rejection. Acute antibody-mediated rejection of organ allografts usually presents as severe dysfunction with a high risk of allograft loss. Peritubular capillary complement C4d deposition with renal dysfunction, associated with circulating donor-specific HLA alloantibodies, heralds organ rejection.<sup>66</sup> Substantial reduction of alloantibody titers followed by suppression of antibody production has become possible with the appropriate use of IVIG therapy and other therapeutic strategies that include combinations of plasmapheresis (or immunoadsorption), tacrolimus, mycophenolate mofetil, rituximab, or splenectomy.<sup>67</sup> However, the optimal dosage for IVIG to treat acute humoral rejection still remains to be defined.

CD20+ MoAb therapy (rituximab) aimed at depleting B cells and suppressing antibody production has been used as rescue therapy in some episodes of steroid- and antilymphocyte-resistant humoral rejection. Plasmapheresis and/or IVIG, in combination with rituximab, have also been used to desensitize selected

high-immunologic-risk patients in anticipation of a previously crossmatch-positive solid organ transplantation.<sup>67</sup> In the near future, the possible role of new specific anti-B-cell approaches or, possibly, of new anti-T-cell activation approaches using selective agents such as belatacept should be assessed to further refine the present treatment of humoral rejection.

In the prophylactic setting (ie, prevention of organ loss in transplant recipients at high immunologic risk), IVIG is associated with reducing the risk of rejection in patients with previous positive complement-dependent cytotoxicity crossmatch.<sup>68</sup> Preformed antibodies to donor HLA or ABO antigens pose a major risk in organ transplantation. Conventional approaches to remove/circumvent this difficulty have included immunoadsorption, plasmapheresis/exchange, splenectomy, and use of cyclophosphamide. Availability of rituximab and IVIG have allowed transplantation in these high-risk patients with reasonable hope of successful outcome.

The ABO-histoblood group system must be considered as transplantation-associated donor-recipient disparity<sup>68</sup> because living donor kidneys from group A donors are now transplanted with increased frequency to group O recipients. This incompatibility was viewed as insurmountable until the advent of a new era of immunosuppressive/tolerance-inducing drugs. Modern genetic blood group typing now allows much more precise typing of histoblood groups, which improves transfusion practice and solid organ transplantation.<sup>69-72</sup> In addition, inhibition of excessive complement activation in organ transplantation might also be ascribed to IVIG preparations, whether enriched or not by IgM.<sup>73</sup> Allografts are given to infants suffering from hypoplastic left heart syndrome but in a recent pilot trial IVIG transfused before and after allograft patching failed to prevent sensitization.<sup>73</sup>

The CMV hyperimmunoglobulins are used both prophylactically and therapeutically. Although some transplantation centers switched to ganciclovir therapy alone, antiviral synergy might occur between ganciclovir and CMV hyperimmunoglobulins. In transplant recipients, CMV may be a risk factor for transplant vasculopathy. Some centers use a combination of the two therapeutic principles: specific viral antibody and virucidal synthetic nucleoside analogues. Prophylactic schedules may include the use of IVIG, rather than IMIG, even in preexposure prophylaxis, such as in the prophylaxis for CMV disease in renal or marrow transplant recipients. Dosage and timing of administration vary. In the postexposure situation, "the sooner the better" is an appropriate attitude. However, preexposure IMIG prophylaxis must be given 2 to 3 days before the exposure, because maximal serum levels of specific antibodies are not reached until 48 to 96 hours after injection.

Animal antibodies against human lymphocytes constitute a series of therapeutic IVIG preparations that are used for patients who have received allogeneic organ transplants.<sup>74</sup> The two major preparations (ATG-Fresenius and thymoglobulin) follow the principle of OKT3 MoAb, to bind to lymphocytes cell surface markers, thereby producing cell lysis or inactivation. Both are

elicited in the rabbit, the first being available in liquid, the second in lyophilized form. The human thymocytes used to immunize the rabbits have been tested for the absence of transfusion-transmitted viruses; the preparations obtained from the rabbits are sterile in this respect. When such preparations are used to treat transplant rejection, they are combined with agents that act in synergistic fashion to provide the potency, avoidance of side effects, convenience of administration, and cost appropriate for the individual patient.

## Coping with Overusage of Intravenous Immunoglobulin Preparations

Access to adequate supplies of IVIG has been a problem in recent years. Expense and limited availability of the product are causing concern for providers and patients alike.

In addition to improved laboratory surveys of Ig therapy, expert panels composed of experienced plasma product prescribers increasingly publish the results of their studies. According to a survey by the Public Hospital Pharmacy Coalition, only half of approximately 350 member hospitals have been able to obtain enough IVIG product to fulfill their patients' needs. That shortage may also be a problem of pricing; one must be aware that with IVIG, increasing usage cannot be offset by increased (bio-) reactor synthesis, as with most drugs, but indeed calls for more plasma donation. Canada was particularly motivated to review expert prescription because its per capita use of IVIG more than doubled from 1998 to 2006. This growth was attributable to off-label usage. To help ensure IVIG use is in keeping with an evidence-based approach to the practice of medicine, the National Advisory Committee on Blood and Blood Products of Canada and Canadian Blood Services convened a panel of national experts to develop evidence-based practice guidelines on the use of IVIG for hematologic conditions. The primary sources used by the panel were three recent evidence-based reviews. Recommendations were based on interpretation of the available evidence and where evidence was lacking, a consensus of expert clinical opinion was employed. A draft of the practice guideline was circulated to hematologists in Canada for feedback. Specific recommendations for routine use of IVIG were made for seven conditions including acquired red cell aplasia, acquired hypogammaglobulinemia (secondary to malignancy), fetal and neonatal alloimmune thrombocytopenia, hemolytic disease of the fetus and newborn, HIV-associated thrombocytopenia, idiopathic thrombocytopenic purpura, and posttransfusion purpura. IVIG was not recommended for use, except under certain life-threatening circumstances, for eight conditions including acquired hemophilia, acquired von Willebrand disease, autoimmune hemolytic anemia, autoimmune neutropenia, hemolytic transfusion reaction, hemolytic transfusion reaction associated with sickle cell disease, thrombotic thrombocytopenic purpura/hemolytic uremic syndrome, and virus-associated hemophagocytic syndrome. IVIG was not recommended for two conditions (aplastic anemia

and hematopoietic stem cell transplantation) and was contraindicated for heparin-induced thrombocytopenia. For most hematologic conditions reviewed by the expert panel, routine use of IVIG was not recommended. Development and dissemination of evidence-based guidelines may help to facilitate appropriate use of IVIG.<sup>75</sup>

## Side Effects Caused by Immunoglobulin Treatment: An Update

IVIG and SCIG administration may be followed by side effects. The incidence of side effects depends on the tolerability of each manufacturer's preparation. Comprehensive reviews of side effects of IVIG reveal few events except for a heterogeneous group of single cases<sup>76,77</sup> (Table 18-2). As with other plasma products, contamination of IVIG and SCIG with infectious agents is a possibility. Until the 1980s it was generally believed that IVIG and SCIG obtained by the Cohn fractionation method could not transmit viruses. However, between 1983 and 1994, more than 200 patients treated with non-virus-inactivated IVIG preparations developed hepatitis C. All manufacturers reevaluated the virus removal and inactivation capacity of their fractionation processes and many added specific pathogen inactivation and/or removal procedures to improve the viral safety of their products. As a result, there have been no further cases of viral transmission with IVIG preparations reported. The risk of infectious antigen transmission is the same for both IVIG and SCIG. Emerging viruses, such as avian influenza A (H5N1) or West Nile virus, are reliably removed by current virus inactivation procedures and are monitored by hemovigilance surveillance.

Prions are proteins responsible for the transmission of Creutzfeldt-Jakob disease (CJD). The variant form of this disease

(vCJD) is of concern because of its shorter incubation period and more severe expression of disease. Blood-borne transmissible spongiform encephalopathy (TSE) infectivity can be undetected for years in the blood of an asymptomatic vCJD individual. Although there is a possibility that vCJD may be transmitted through whole blood transfusions, there have been no reported cases of prion transmission through plasma-derived products.<sup>79,80</sup> This may be because the prions are removed during the fractionation process and the removal can be enhanced by depth filtration, adsorption chromatography, and nanofiltration (Roemisch J, personal communication). Other methods of prion removal have been investigated. The recently founded company, Pathogen Removal and Diagnostic Technologies, Inc, has developed a strategy to select resins that best adsorb the TSE causative agent from blood, although it is still experimental. The company has observed that selected resins can remove as much as 5–6 log<sub>10</sub> PrP<sup>Sc</sup>, if not up to 8 log<sub>10</sub>.

Reported reactions to SCIG include dizziness, malaise, chills, nongeneralized skin reactions such as swelling, redness, induration, and/or soreness.<sup>82</sup> Most of these reactions do not result in discontinuation of the SCIG treatment. A final viscosity of >10 centipoise for SCIG preparations increases the probability of local reactions. Although not routinely performed, IgA antibodies can be screened using standard particle gel immunoassay technique (DiaMed-AG, Cressier sur Morat, Switzerland) or ELISA methods.<sup>83,84</sup> It appears that SCIG is less prone than IVIG to induce anaphylactoid reactions upon IgA-anti-IgA immune complex formation.

The continuous improvement of purification and virus inactivation and removal by major manufacturers of IVIG and SCIG preparations has contributed to containment of side effects. The use of caprylate and nanofiltration are relatively newer methods that have been employed.

**Table 18-2.** The Adverse Event Spectrum Shared by Intravenous and Subcutaneous Immunoglobulins

| Adverse Event                            | IVIG*                                                                                                                                              | SCIG                                                        |
|------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------|
| <b>Time after administration</b>         |                                                                                                                                                    |                                                             |
| Immediate                                | Anaphylaxis (rare)                                                                                                                                 | None so far                                                 |
| Prolonged                                | Hyperviscosity inducing thromboembolic events and clogging of microcirculation, <sup>78</sup> urticaria, aseptic meningitis, hemolysis, leukopenia | Swelling, induration and/or soreness, redness               |
| Delayed                                  | Infectious disease transmission, <sup>†</sup> PrP infection <sup>‡</sup>                                                                           | Infectious disease transmission, PrP infection <sup>‡</sup> |
| <b>Causative agent or property</b>       |                                                                                                                                                    |                                                             |
| Stabilizers                              | Renal insufficiency                                                                                                                                | None                                                        |
| Low temperature of IVIG/SCIG preparation | Critical in cold agglutinin disease to induce hemolysis <sup>81</sup>                                                                              | Room temperature in any patient                             |
| IgA contamination                        | Anti-IgA carriers                                                                                                                                  | Not (yet?) described                                        |

\* See Nydegger and Sturzenegger<sup>76</sup> and Katz et al.<sup>77</sup>

<sup>†</sup>During the last 10 years no further reports of transmission because of stringent requirements issued by regulatory agencies.

<sup>‡</sup>Theoretically possible, to date never reported; see Aguzzi and Glatzel<sup>79</sup> and Foster.<sup>80</sup>

## Summary

Immune globulins as therapy have progressed significantly and have the potential for many more applications. New indications could result in a huge change in the requirement for this product. Further clinical trials and development for targeted antibodies suggest a complex but significant future for this form of therapy.

## Acknowledgments

The author acknowledges continuous support of Octapharma, Switzerland, and Labormedizinisches Zentrum Dr. Risch, AG, Liechtenstein.

## Disclaimer

The author has disclosed professional relationships with Octapharma and Biotest.

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# 19

## Preparation of Plasma Derivatives

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Plasma derivatives prepared from human plasma play a major role in the therapy of many diseases. Historically they have been used primarily in critical care settings and in the treatment of inherited diseases. However, in recent years intravenous immune globulin (IVIG), like therapeutic plasma exchange, has been used to treat a variety of diseases—usually with some type of connection to the immune system. Demand for the products has increased. For some preparations (such as Factor VIII and IX) alternatives exist in recombinant products; but for other products, no replacement appears on the horizon. Thus, this highly specialized area of medical therapy plays an important role in the care of many patients.

### Evolution of the Field

Derivatives of plasma for infusion into humans are developed through the fractionation of plasma. The scientific development of this field began in the laboratories of Edwin Cohn at Harvard University in the 1930s. Using ethanol, he developed a method that allowed the purification of albumin. Additional fractions were found to include other proteins of potentially clinical significance. With war on the horizon, the United States Navy took the lead in organizing studies and supporting Cohn's work, and partnerships with industry were developed.<sup>1</sup>

Albumin was sought as an alternative to freeze-dried plasma to treat battlefield casualties in shock. The most available source was bovine albumin. Early studies in volunteers (medical students at Harvard) had not demonstrated hypersensitivity. But when the bovine albumin was administered in clinical quantities to prisoner volunteers, serum sickness developed in a high proportion of the inmates, and one death occurred. Therefore, attention was focused on producing large quantities of human

albumin. Human albumin was taken to Hawaii after the attack on Pearl Harbor and used successfully to treat burn victims. In 1942 a National Research Council conference on albumin determined it was of value in shock and burns and found further advantages. Albumin could be packaged in one-tenth the space required for freeze-dried plasma, was ready in an emergency without reconstitution, and remained stable at room temperatures and even high outdoor temperatures. The American Red Cross was charged with collection of blood for further manufacture into this derivative, and contracts with pharmaceutical plants were arranged to ensure optimal production. Thus began the field of plasma fractionation, with its first phase dominated by albumin.<sup>1</sup>

Not immediately appreciated was the safety of albumin with regard to virus transmission. Pasteurization of the product from the beginning rendered it safe. At the time there was a lack of appreciation for the potential value of saline solutions, which awaited further research. Most of the blood collected during the war years was for albumin and the red cells were often discarded.

Albumin utilization continued after the war. Soon the demand for albumin could not be met by Recovered Plasma (plasma obtained as a by-product after separation and processing of whole blood into plasma and cellular components). The discovery of plastic bags, additive solutions, and the routine separation of whole blood into red cells and plasma allowed the development of increased Recovered Plasma collections. In the United States the use of plastic bags instead of glass bottles for blood collection facilitated emergence of an industry parallel to the field of blood banking to collect Source Plasma (plasma collected specifically for further manufacture). With the development of automated plasmapheresis devices for the collection of Source Plasma, accidents caused by returning red cells to the wrong donor could be completely avoided. Automated plasmapheresis was not only safer but also quicker and more comfortable for the donor; thus, Source Plasma establishments expanded. These organizations were usually for-profit companies, either independently owned or part of a company that also fractionated the plasma, and paid their donors in cash for

the donation. Initially, this did not differentiate them from many blood banks in the United States that also paid their donors until the all-volunteer system emerged in the United States in the 1970s.

Judith Poole's discovery of cryoprecipitation led to the development of antihemophilic factor. When home care started to be used extensively to treat patients with hemophilia A, the demand for clotting factor became the principal driver for plasmapheresis (a second phase in the field from approximately 1970 to 1990). When recombinant factor products appeared on the horizon, many predicted the end of the industry. However, the discovery of methods to manufacture an immune globulin product that could be given intravenously without precipitating or activating complement resulted in a third phase (1990 to the present)—characterized by immune globulin products as the principal driver of the industry.<sup>2</sup> Table 19-1 shows the products produced by the plasma industry. The composition of plasma is outlined in Table 19-2 and described in detail in Chapter 17.

## Plasma Procurement

Recovered Plasma is the plasma obtained after processing of whole blood into red cell and plasma components, while Source Plasma is the plasma obtained by apheresis after all the red cells and almost all of the white cells have been returned to the donor and is intended for further manufacture into injectable or diagnostic products. Availability of Recovered Plasma depends on collection of whole blood, which is driven by the demand for red cell components. The supply of Recovered Plasma is not sufficient to meet the demand for plasma derivatives; in fact, its current availability may be decreasing as a result of trends in red cell utilization and collection by apheresis. Thus, Source Plasma collections are needed to meet patient needs and the related market demands. Rapid changes in the market can be absorbed by opening and, if necessary, closing centers and increasing or decreasing donation numbers to meet actual need for the products. Although whole blood donors are limited to four to six donations per year depending on local regulations, Source Plasma donors can donate up to 104 times per year in the United States and up to 40 times per year in Europe. Thus, a Recovered Plasma donor can provide 1.5 liters of starting material per year, while a Source Plasma donor can provide almost 90 liters in the United States and approximately 25 liters in Europe. The concentration of Factor VIII is higher in Source Plasma (about 100 U/dL) than in Recovered Plasma (66-84.5 U/dL), but IgG is higher in Recovered Plasma (9.4 g/L compared to 8.2 g/L). Clotting Factor VIII is an acute-phase reactant not lowered by serial plasmapheresis. This attribute, combined with immediate freezing possible in a fixed site, facilitates the improved recovery. In addition, the immediate decrease in pH seen initially with whole blood collection is not seen with plasma collected by apheresis because of the continuous mixing and the lower concentration of anticoagulant (plasma to citrate ratios of 1:16 for Source Plasma

compared to 1:7 for Recovered Plasma). In addition, the release of proteolytic enzymes that destabilize clotting proteins occurs less often with plasmapheresis. When whole blood is drawn for red cells and Recovered Plasma, the collection should ensure good mixing, a short duration (ideally 15 minutes or less), and avoidance of temperature variation. The higher serum protein levels in Recovered Plasma are thought to relate to the less frequent donations.

In recent years, 63.5% of the plasma collected for fractionation has been Source Plasma and the remainder Recovered Plasma. About one-half of the plasma comes from North America, where the ratio of Source Plasma to Recovered Plasma is about 80:20. About one-quarter of the plasma comes from Europe, which accounts for half of all the Recovered Plasma. In the United States, there were between 10 and 13.8 million plasmapheresis collections per year between 2002 and 2006. More specifically, there were approximately 12.4 million plasmapheresis collections in 315 centers in the United States in 2006. As the number of plasmapheresis donor centers has declined from more than 400 in 2002, the collections per center have increased by about 16% with a mean of more than 39,000 collections per center annually.

The plasma industry has been a global one for many years. The plasma fractionation industry is considered part of biologics manufacturing, which, in turn, is a subset of the pharmaceutical industry. Intermediate preparations might be moved from a plant in one country after initial production steps to another plant in another country for completion of manufacture. Products are typically registered in multiple countries, although products sold in the United States must come from either plasma collection establishments in the United States or facilities overseas that are licensed by the US Food and Drug Administration (FDA). However, in some countries, particularly in Western Europe, national fractionation plants owned by nonprofit (tax exempt) entities remain. In all cases, the plant must use aseptic conditions, be in conformance with good manufacturing practice (GMP) regulations, and generally be licensed by regulatory authorities of all countries that receive products produced there. Similarly, United States plasma donor centers must be approved by European authorities if their plasma is sent to Europe.

The production of plasma derivatives (also known as biologics, biopharmaceuticals, or biological therapies) differs from standard pharmaceutical manufacturing in several respects. The cost of the starting material is high, reportedly 45% to 50% of the cost of these products, whereas the cost of the raw material for standard pharmaceuticals averages 19% of the total. Thus, the cost of procurement of plasma plays a key role in the economics of the industry. Exclusive use of nonremunerated donors remains a major goal of international and European organizations. However, the volume of Recovered Plasma for fractionation has declined by about 14% in the last 5 years, and the volume of Source Plasma from remunerated donors in the United States and donors compensated for time and transportation expense in Germany, Austria, and China has increased by about 4%.

**Table 19-1.** Clinically Used Plasma Products

| Plasma Product                                                                     | Preparations                                                                  | Indications (Usage)                                                                                                                                          |
|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Albumin</b>                                                                     |                                                                               |                                                                                                                                                              |
| Human serum albumin                                                                | 4-5% or 20-25% solution for intravenous injection                             | Severe acute hypoproteinemia<br>Exchange transfusion in neonates<br>Adult respiratory distress syndrome                                                      |
| Plasma protein fraction                                                            | 4.5-5% solution for intravenous injection                                     | Shock syndromes<br>Severe burns<br>Therapeutic plasma exchange                                                                                               |
| <b>Immunoglobulins</b>                                                             |                                                                               |                                                                                                                                                              |
| Multispecific (normal)<br>Immune serum globulin                                    | Freeze-dried or in solution (16%) for intramuscular injection                 | Hypo-/agammaglobulinemia<br>Prophylaxis of infectious diseases (hepatitis A)                                                                                 |
| Immune globulin intravenous                                                        |                                                                               | Hypo-/agammaglobulinemia<br>Idiopathic thrombocytopenia<br>Kawasaki syndrome                                                                                 |
| Specific (hyperimmune)<br>Cytomegalovirus (CMV)<br>immune globulin                 | Freeze-dried or in solution for intravenous injection (6%)                    | Prevention of:<br>CMV infection in immunosuppressed individuals<br>HBV infection                                                                             |
| Hepatitis B (HBV) immune globulin                                                  |                                                                               | Whooping cough infection                                                                                                                                     |
| Pertussis immune globulin                                                          |                                                                               | Rabies infection                                                                                                                                             |
| Rabies immune globulin                                                             |                                                                               | Hemolytic disease of the fetus and newborn and D sensitization in other situations                                                                           |
| Rh immune globulin                                                                 |                                                                               | Tetanus infection                                                                                                                                            |
| Tetanus immune globulin                                                            |                                                                               | Smallpox infection                                                                                                                                           |
| Vaccinia immune globulin                                                           |                                                                               | Chickenpox infection                                                                                                                                         |
| Varicella immune globulin                                                          |                                                                               |                                                                                                                                                              |
| <b>Coagulation Proteins</b>                                                        |                                                                               |                                                                                                                                                              |
| Factor VIII<br>Factor IX complex                                                   | Freeze-dried, for intravenous injection (potency data vary for manufacturers) | Hemophilia A<br>Hemophilia B, Factor VII-, Factor X-, Factor II-deficiency, warfarin overdose                                                                |
| Factor IX<br>Factor VII<br>Factor XIII<br>von Willebrand factor<br>Cryoprecipitate |                                                                               | Hemophilia B<br>Factor VII deficiency<br>Factor XIII deficiency<br>von Willebrand disease<br>Hemophilia A<br>von Willebrand disease<br>Fibrinogen deficiency |
| Activated Factor IX complex/Factor VIII by-passing activity                        |                                                                               | Factor VIII inhibitor                                                                                                                                        |
| Fibrin sealant                                                                     | Freeze-dried, for topical application                                         | Wound healing                                                                                                                                                |
| <b>Protease Inhibitors</b>                                                         |                                                                               |                                                                                                                                                              |
| Antithrombin<br>Alpha-1-proteinase inhibitor<br>C1-esterase inhibitor              | Freeze-dried, for intravenous injection (potency data vary for manufacturers) | Antithrombin deficiency<br>Proteinase inhibitor deficiency<br>Hereditary angioneurotic edema                                                                 |

Table 19-2. Constituents of Plasma for Fractionation

| Constituent                                     | Amount (g/L) |
|-------------------------------------------------|--------------|
| <b>Major</b>                                    |              |
| Albumin                                         | 30.0 to 40.0 |
| Immune globulins                                | 6.0 to 19.0  |
| Fibrinogen                                      | 2.5 to 3.5   |
| Transferrin                                     | 2.0 to 3.0   |
| Alpha 1-proteinase inhibitor                    | 1.5 to 2.0   |
| Alpha 2-macroglobulin                           | 1.3 to 2.0   |
| Complement factor C3                            | 1.0 to 1.8   |
| <b>Minor</b>                                    |              |
| All other proteins (eg, Factor VIII, 0.2 mg/L)  | <0.1         |
| <b>Total protein</b>                            | 60.0         |
| (approximately 120 well-characterized proteins) |              |

Remunerated donors from the United States have proven to be safe and dependable. They are the key group of donors in allowing the industry to respond to patient needs.<sup>3</sup>

More detail on recruitment, collection, and processing of donors for plasma is covered in Chapter 62.

## Plasma Manufacture

### Shipping Process

The overall process begins with the shipping of collected units once all testing has been completed and all information verified. Plasma stored at refrigerated (and even room) temperatures can be used for some products (albumin and immune globulins). But the efficacy of the process and the assurance of cost-effective production from the starting material require that labile clotting factor concentrates be made from each liter. Thus, plasma stored frozen at  $-20$  to  $-30^{\circ}\text{C}$  is the standard starting material. Frozen plasma also reduces issues of bacterial growth during shipment and storage. Plasma is held for 60 days after collection as part of the safety procedures (described below). After that time, the process begins under aseptic conditions in a manufacturing plant.

### Manufacturing Process

The manufacturing process has been reviewed in detail elsewhere.<sup>4,5</sup> The methods of large-scale plasma protein manufacturing are described in Table 19-3.

The plasma is expelled from containers and thawed at  $4^{\circ}\text{C}$  to produce cryoprecipitates. Typical batches are 2000 to 4000 L. Only those precipitation and other separation methods that do not lead to denaturing or inactivation of proteins are used. The basic Cohn fractionation procedure is shown in Fig 19-1 and

Table 19-3. Methods of (Large-Scale) Plasma Protein Fractionation

|                                                                     |
|---------------------------------------------------------------------|
| <b>Differential solubility</b>                                      |
| Cryoprecipitation                                                   |
| Salting-out with neutral salts or amino acids                       |
| Neutral polymer precipitation                                       |
| Organic solvent-aqueous systems                                     |
| Soluble anionic precipitants                                        |
| <b>Differential interaction with solid media</b>                    |
| Generalized adsorption onto surfaces                                |
| Ion exchange (including polyelectrolyte interaction) chromatography |
| Immune affinity chromatography                                      |
| Hydrophobic interaction chromatography                              |
| Ultrafiltration                                                     |
| Charged membrane interaction                                        |
| Adsorption membrane interactions                                    |
| Gel filtration                                                      |
| <b>Differential interaction with physical fields</b>                |
| Centrifugal techniques                                              |
| Electrophoretic techniques (limited scale)                          |
| Differential thermal denaturation                                   |

the relationship of the fractions to the products are described in Table 19-4. Figure 19-2 provides an overview of the manufacturing process for major products.

Methods for plasma fractionation are based on the principles of differential solubility of proteins and differential interaction of proteins with solid media. Differential interactions with physical fields have been used only rarely. The solubility of proteins is determined by charge, isoelectric point, size, composition, conformation, and physical and chemical properties. The cold-ethanol precipitation developed by Cohn<sup>6</sup> (later modified by Oncley<sup>7</sup> and Kistler and Nitschman<sup>8</sup>) induces changes in protein solubility to isolate various fractions. Successive processing steps are carried out at specific ethanol concentrations and pH, temperature, and osmolality. This results in selective precipitation of proteins, which are then separated by centrifugation and/or filtration.

The Cohn method uses five variables: ethanol concentrations ranging from 8% to 40%, pH levels between 4.5 and 7.4, temperature ranges from  $-5^{\circ}$  to  $-7^{\circ}\text{C}$ , ionic strength differentials from 0.14 to 0.01, and protein concentrations from 5.1% to 0.8%. As the ethanol concentration or pH is changed, different protein fractions are obtained. Removal of ethanol is by freeze-drying or ultrafiltration.

Chromatographic techniques were introduced in the 1960s through 1980s. One use was to isolate cryoprecipitates that were recovered using continuous centrifugation, with the precipitate recovered from the bowls and frozen at  $-30^{\circ}\text{C}$  or colder until processed into Factor VIII. The techniques were also used to increase IgG recovery and remove viruses.

Anion exchange chromatography and affinity chromatography using immobilized heparin or monoclonal antibodies

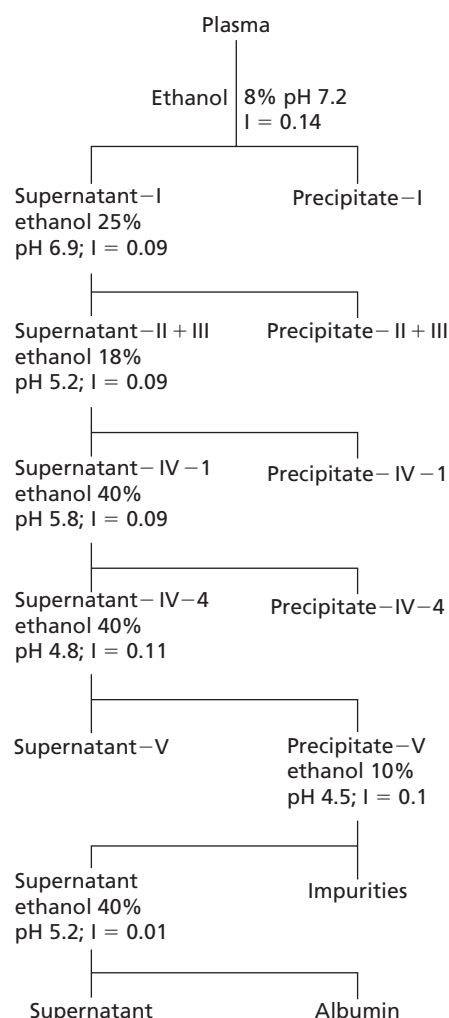


**Figure 19-1.** Plasma fractionation scheme showing one of the original schemes used by Cohn et al.<sup>6</sup>

(immunoaffinity chromatography) are examples of chromatographic techniques used. Chromatography is capable of improving purity, extracting trace labile proteins, optimizing protein recovery, and removing virus inactivation agents. It is used increasingly for anti-D production.

The principal advantage of the cold-ethanol or Cohn method is that it facilitates large-scale production in batches that can be traced. Ethanol is readily removed, bacterial growth is inhibited throughout the process, and materials are inexpensive. Ethanol's long history has created a comfort level with manufacturers and regulators alike. But it is unsatisfactory for recovery and maintenance of function of some proteins. Thus, the various newer chromatographic techniques have been essential for the field to move forward. The advantages of these techniques are selectivity and high yield. The disadvantages are the requirements for larger processing volume for the dilute proteins, potential for bacterial growth during processing, inability to handle high lipid content (degrading of the columns), and lost ligand from the columns (leaching).<sup>4</sup>

**Table 19-4.** Cohn Fractionation Method and Distribution of Plasma Proteins

| Fraction | % Ethanol | pH  | Proteins                                                                                                                                      |
|----------|-----------|-----|-----------------------------------------------------------------------------------------------------------------------------------------------|
| I        | 8-10      | 7.2 | Fibrinogen, fibronectin, Factor VIII, complement factors C1q, C1r, and C1s                                                                    |
| II + III | 25        | 6.9 | Factors II, V, VII, IX, and X, fibrinogen, immunoglobulins G, A, and M; $\alpha$ - and $\beta$ -globulins; $\beta$ -lipoproteins; plasminogen |
| IV-1     | 18        | 5.2 | Antithrombin, $\alpha$ -1-proteinase inhibitor, complement components, immunoglobulin M, ceruloplasmin, $\alpha$ -lipoprotein, albumin        |
| IV-4     | 40        | 5.8 | Transferrin, haptoglobin, ceruloplasmin, $\alpha$ - and $\beta$ -globulins, $\alpha$ -lipoprotein, albumin                                    |
| V        | 40        | 4.8 | Albumin, $\alpha$ - and $\beta$ -globulins                                                                                                    |

### Factor VIII and von Willebrand Factor

The initial step in the production of antihemophilic factor or Factor VIII is cryoprecipitation. When the frozen plasma is thawed rapidly at 4°C, certain proteins are not dissolved, thus forming the precipitate that contains 50% or more of the Factor VIII in the original unit. Before extensive use of Factor VIII concentrates, this cryoprecipitate (when dissolved in a small amount of saline or plasma) was used as a blood bank component for therapy. It has the advantage of a very high concentration of the coagulation factor.<sup>9</sup>

Further purification, as shown in Fig 19-3, leads to the Factor VIII concentrate. The cryoprecipitate is typically subjected to a combination of aluminum hydroxide adsorption and precipitation or precipitation using glycine. This further removes traces of the other coagulation factors, particularly fibrinogen, which is also concentrated (40% of the starting fibrinogen in the cryoprecipitate). At this stage, the product contains all multimers marking the Factor VIII:Ag and the von Willebrand factor (vWF). It can be used to treat von Willebrand disease when desmopressin (DDAVP) treatment alone is ineffective or contraindicated or to manage acquired Factor VIII deficiency especially involving antibodies against Factor VIII (see Chapter 28).

In the late 1980s, further purification steps were introduced using the immunoaffinity technique and monoclonal antibodies. These methods led to a highly purified Factor VIII concentrate (without vWF) with a specific activity of 100 to 250 IU/mg of protein. This product is not suitable for treatment of von Willebrand disease, but is effective in patients with hemophilia A.

### Fibrinogen

Fibrinogen is prepared through the use of multiple plasma or cryoprecipitate precipitation steps using ethanol or glycine or is purified by further steps from fibronectin. Fibrinogen is mainly used in massive blood losses after severe trauma, obstetric complications with bleeding, tumors with disseminated intravascular coagulation (DIC), or lack of fibrinogen production because of liver disease (see Chapter 24). Currently, fibrinogen is not

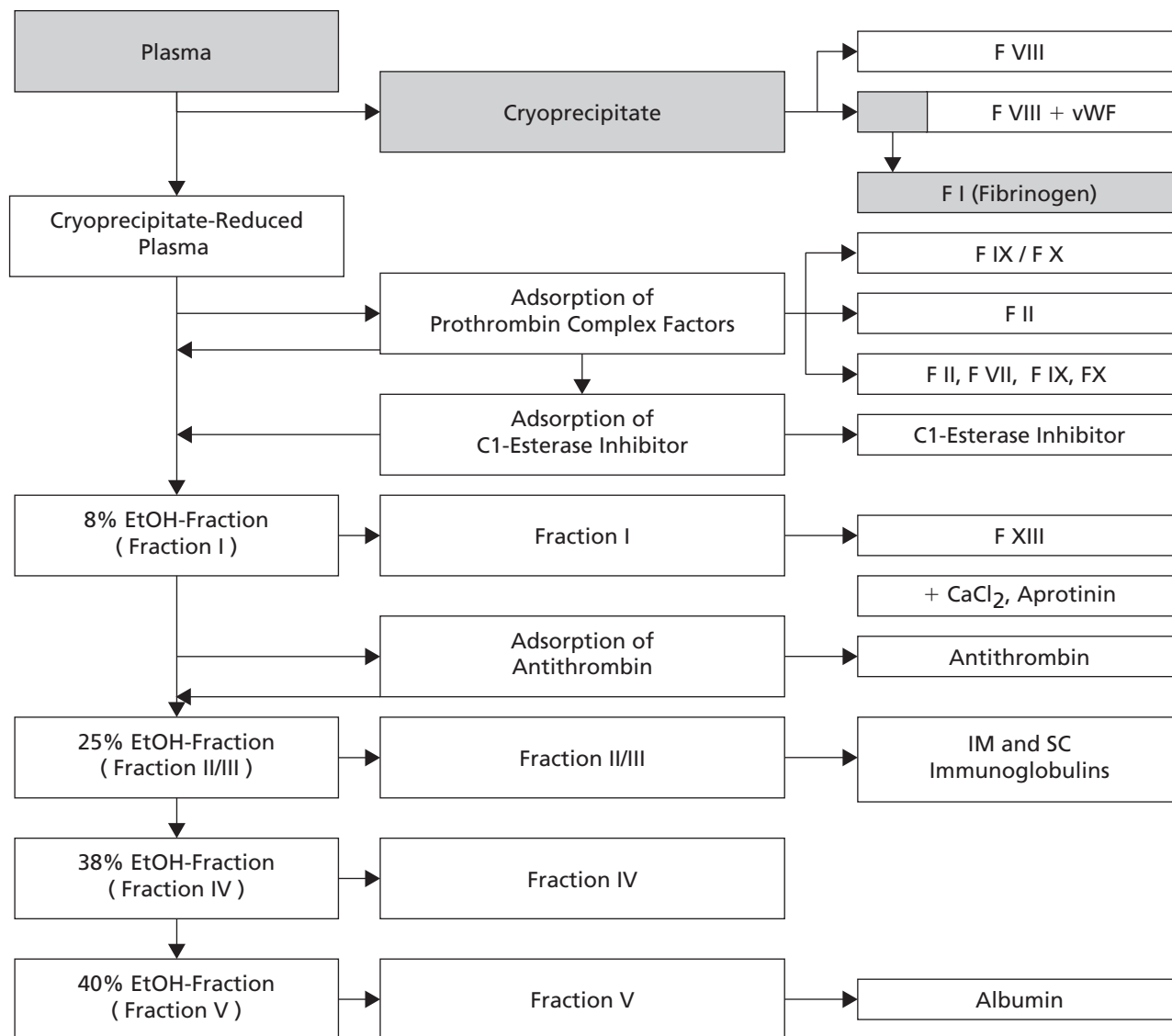


**Figure 19-2.** A simplified plasma fractionation scheme showing progression of plasma to produce the primary products. vWF = von Willebrand factor; EtOH = ethanol; IM = intramuscular; SC = subcutaneous.

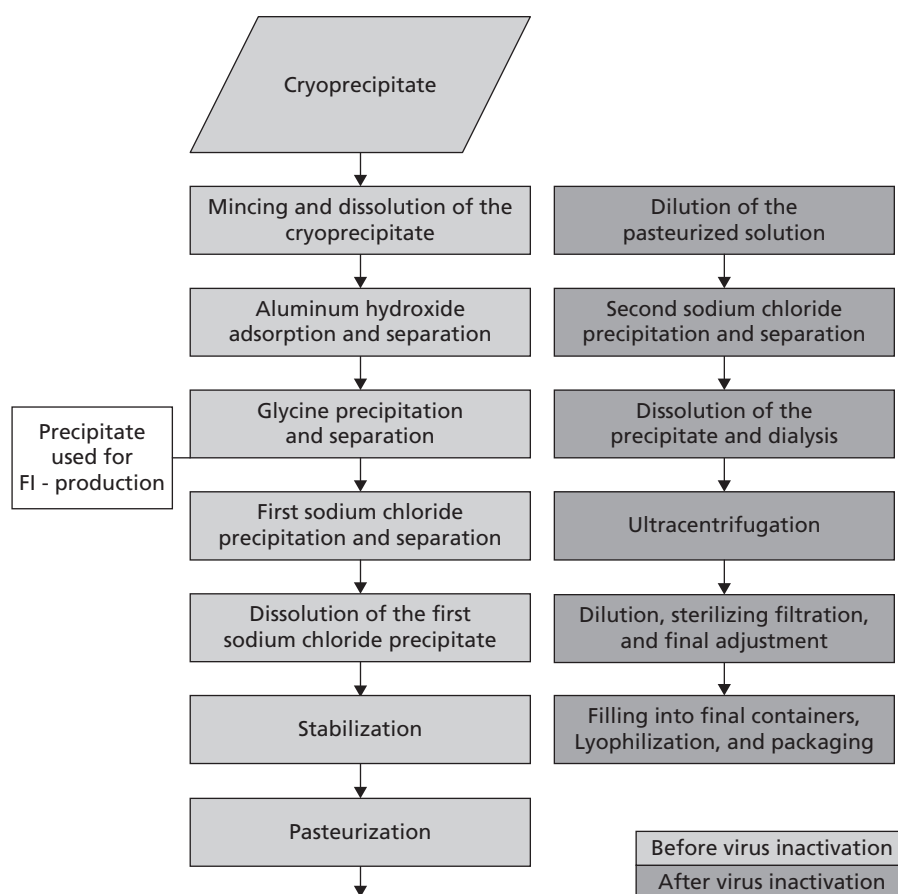


**Figure 19-3.** A sample fractionation scheme to produce a pasteurized Factor VIII concentrate beginning with cryoprecipitation.

available in the United States but is available in Europe as a licensed product.

### Fibrin Sealants

Fibrin sealants (glues) are thrombin-fibrinogen concentrates and are used topically to induce hemostasis, sealing, and healing. The fibrinogen comes from Cohn Fraction I or cryoprecipitate and generally is formulated to contain fibronectin, vWF, plasminogen, and/or Factor XIII. It is used increasingly in release systems for antibiotics, growth factor, and cytostatic medications.

### Prothrombin Complex and Other Clotting Factors

Initially developed as a mixture of vitamin-K-dependent factors (Factor II, VII, IX, and X), prothrombin complex concentrate is formulated by adsorption to anion exchangers. The final product contains heparin to avoid activation of clotting factors. In addition, some concentrates contain small amounts of antithrombin. In the late 1980s, purified Factor IX products were developed. The production process uses advanced biotechnology consisting of immunoaffinity chromatography that precisely targets and separates the Factor IX protein through the use of specific monoclonal antibodies. Similar processes have been used to purify

Factor VII, XI, and XIII. Factor XIII is used increasingly for patients with wound healing problems or with congenital disorders causing vascular diathesis or collagen defects. Factor VII and XI are not in widespread use. The Factor II-VII-IX-X complex product has been purified to reduce thrombogenic potential. It is now available in Europe and is undergoing clinical trials in the United States for reversing warfarin anticoagulation and treating liver disease.

### Antithrombin

Antithrombin (AT) concentrates are extracted from the 8% ethanol supernatant of cryoprecipitate-reduced plasma by affinity chromatography and adsorption in batch procedures. Other proteins are removed by washing with complex-forming solutions. The AT is then eluted, stabilized by saccharose and glycine, and pasteurized.

Inherited AT deficiency is associated with an increased risk of thromboembolic events. Prophylactic AT therapy is given to such adult patients typically during surgery or childbirth or to newborn infants during the perinatal period. The aim of treatment is to achieve AT activity of at least 80%.

Acquired AT deficiency can result from an increased consumption of AT such as with DIC or sepsis, reduced production



of AT associated with liver disorders, or an increased loss of AT seen in burn injuries and multiple trauma injuries. The usefulness of this concentrate in acquired AT deficiency has been challenged recently.<sup>10</sup>

### **Alpha<sub>1</sub>-Proteinase Inhibitor**

This product (also known as alpha<sub>1</sub>-antitrypsin inhibitor) can be isolated from Fraction IV by polyethylene glycol (PEG) precipitation and ion exchange chromatography or from supernatant Fraction II + III, where its extraction competes with albumin. It is used for patients with an inherited deficiency of the inhibitor that results in severe emphysema and liver disease. A recent study has shown that large-scale manufacturing results in structural heterogeneity among the products, but there was no demonstrated impact on therapeutic efficacy.<sup>11</sup>

### **Protein C**

The dominant concentrate of protein C in the United States is produced by recombinant technology in established human cells and activated by transfer into bovine cells. Its main function is the downregulation of the clotting cascade by splitting of the clotting Factors VIIa and Va.

### **C1-Esterase Inhibitor**

This component is collected from cryoprecipitate-reduced plasma after extraction of Factors II, VII, IX, and X and purified by different precipitation and filtration steps. Individuals lacking this inhibitor are subject to hereditary angioedema.

### **Immunoglobulins**

Immunoglobulin products, covered in more detail in Chapter 18, are obtained from the 25% ethanol fraction. Human immunoglobulin for intravenous and subcutaneous administration is indicated for immunodeficiencies and a variety of other conditions. The manufacturing process begins with dissolution of Fraction II/III in glycine followed by precipitation of accompanying proteins and inactivation of viruses. After addition of dicalite via multilayer filters, further purification steps to improve recovery are employed. Total chromatographic procedures are used for specific immunoglobulin preparations such as Rh Immune Globulin (RhIG).

Special immunoglobulins [hyperimmune globulins against cytomegalovirus, rabies, hepatitis A virus (HAV), hepatitis B virus (HBV), tetanus, varicella] are liquid preparations for intramuscular injection that contain a standardized amount of antibody to the specific infectious agent. The Source Plasma comes from either 1) donors screened for the specific antibodies with the relevant donations sorted and stored for a special fractionation pool or 2) compensated donors who have been immunized with specific commercially available antigens (vaccines). The latter is now the more common method and is termed hyperimmunization. It can achieve exceptionally high antibody levels for collection of what is termed "hyperimmune plasma," especially if the vaccine labeling permits frequent vaccination. Hyperimmune plasma is then added together in one fractionation pool for

production. These immunoglobulins are used in the treatment of patients at high risk but without adequate immunity for one of the above-mentioned infections. For example, rabies immune globulin treatments are lifesaving for a person bitten by a rabid animal who had not received the rabies vaccine. The immune globulin provides protective antibody before the individual completes the series of vaccinations that will provide this immunity.

Anti-D or RhIG is obtained from plasma from individuals sensitized to the D antigen or volunteers injected with specially matched and screened Rh-positive red cells. RhIG is used to treat Rh-negative females who through transfusion or pregnancy may acquire Rh antibodies that might harm future children by causing hemolytic disease of the fetus and newborn (see Chapters 26 and 27).

### **Albumin**

The Cohn cold-ethanol procedure was originally designed to prepare albumin. With depth filtration, relatively high-concentration and high-purification albumin is obtained. Nevertheless, some methods use ion exchange chromatography, anion exchange, cation exchange, and size exclusion chromatography as well. Concentration is adjusted by ultrafiltration.

Albumin represents two-thirds of the proteins in plasma. Albumin deficiency, seen in renal disease with increased protein loss and in liver disease with decreased protein production, leads to edema and circulatory issues because of diminished oncotic pressure in the circulation. Thermal injury also leads to loss of albumin. Current indications for albumin administration are discussed in Chapters 20 and 39.

### **Virus Inactivation/Removal**

Following the appearance of human immunodeficiency virus (HIV) transmission by transfusion of blood components and plasma derivatives, a major portion of scientific activity in the plasma industry has been directed at either inactivation (such as pasteurization developed by Norbert Heimburger<sup>12</sup> or solvent/detergent treatment developed by Bernard Horowitz<sup>13</sup>) or removal of viruses to ensure product safety. By 1994, the industry had largely achieved this goal, as indicated by the absence of disease transmission of hepatitis or HIV by any product manufactured by American or European biological producers. In fact, no antihemophilic product has transmitted disease since 1987. After the introduction of second-generation hepatitis C virus (HCV) antibody testing, the resulting loss of protection from the antibody in immune globulin products led to transmission of HCV. The introduction of dedicated virus-inactivation methods into the manufacturing process of IVIG has prevented such transmission from occurring again.<sup>14</sup>

The success of virus inactivation/removal requires not only validation of the reduction in viral load but also maintenance of the intact therapeutic protein. Techniques such as electrophoresis (especially capillary electrophoresis), size exclusion gel chromatography, isoelectric focusing, or antigen/activity ratio are needed. In addition, nonimmunogenicity must be demonstrated

to ensure that neoantigens have not been introduced. Amino acid analysis, cleavage with proteolytic enzymes, circulatory survival in animal models, and measurement of sedimentation and diffusion coefficients, viscosity, circular dichroism, and optical rotary dispersion are used.<sup>15</sup>

Product safety ultimately is achieved by the combination of donor selection, testing of donations and plasma pools, and virus inactivation and removal in the manufacturing process, demonstrated in virus validation studies. The virus validation studies must be performed in dedicated laboratories separate from manufacturing facilities so as not to contaminate the production facility. A process step that removes 4 logs or more of virus is considered effective when the process step can be reliably performed and is insensitive to modifications within the specifications of the manufacturing process.

Viruses chosen for validation studies should closely resemble the viruses that may potentially be present in contaminated plasma, ie, transfusion-relevant viruses. To test the ability of the production process to remove viruses in general, they should also represent a wide range of physicochemical properties. For these studies, strains of viruses should be chosen that replicate to high titers in cell culture and can be assayed in an effective, sensitive, and reliable manner in in-vitro infectivity assays. Routine infectivity assays are currently not available for HBV, HCV, or parvovirus B19. A research infectivity assay for parvovirus B19 has been established in different laboratories and parvovirus neutralization during manufacturing has recently been reported.<sup>16</sup> Consequently, these actual viruses cannot be used for virus validation studies and, where appropriate, model viruses are used instead.

Virus validation studies should be performed with HIV and HAV as relevant viruses. For those blood-borne viruses that cannot be propagated in cell culture systems, model viruses must be used. Model viruses for HCV include, for example, bovine viral diarrhea virus (a flavivirus) or Sindbis virus (a togavirus). A specific model virus for HBV is not available; therefore, nonspecific model viruses must be used such as herpesviruses, pseudorabies virus, or, potentially, duck HBV. A wide range of viruses must be used in virus validation studies. For parvovirus B19, currently only experimental cell culture systems are available; therefore, animal parvoviruses (canine or porcine parvoviruses) are used as model viruses. In case of interaction of human immunoglobulins with viruses used in virus validation studies, the use of model viruses may be essential: HAV can be replaced by porcine or bovine enteroviruses or encephalomyocarditis virus.

The virus reduction factor is determined as the difference of the virus load (virus titer  $\times$  volume) in the spiked starting material and in the final sample. In order to demonstrate a high virus reduction factor, it is often appropriate to detect low virus concentration, ie, the largest practical sample size should be evaluated for residual virus infectivity. However, the impact of potential cytotoxicity or interference of test matrix (composition of product intermediate) on the accurate determination of a virus titer has to be evaluated and taken into account in the calculation of the virus reduction factor.

In virus validation studies, selected steps (stages) of the manufacturing process are employed to assess how effectively they could remove a range of viruses. These experiments are based on a scaled-down version of the purification process, which is validated to mimic closely the production-scale manufacturing process. Virus validation studies are performed by deliberately adding cell-culture-derived stocks of either blood-borne viruses, or appropriately selected model viruses, to product intermediates derived from actual production lots. The amount of virus infectivity removed or inactivated by the stage or subsequent stages of the manufacturing procedure was demonstrated in virus validation studies. The extent of virus removal/inactivation at each individual stage or combination of stages of the production process should be tested at least twice, independently. The results of the virus validation studies (in scaled-down procedures) are predictive for production scale, as the laboratory scale was validated with regard to the comparability of the laboratory scale (model) and full scale (manufacturing), demonstrated in scaled-down validation studies. The use of a wide range of viruses in these validation studies also provides indirect evidence that the production process can inactivate or remove any novel or unpredictable virus contamination effectively.

In addition to providing an accurate reflection of the full-scale manufacturing procedure, scaled-down versions of the production process are also used in so-called “robustness” studies. These robustness studies evaluate the production parameters that may have an influence on virus inactivation and/or removal. These include parameters such as concentration of protein and other components such as precipitating agents, temperature, pH, reaction time, amount of stabilizer, temperature for the pasteurization step, and the amount of solvent or detergent in solvent/detergent (SD) treatments. Furthermore, for chromatographic steps, protein concentration, flow rate, washing and elution volume, and resin reuse should be assessed. These robustness studies are performed in the laboratory using parameters in the range of, or even beyond the specifications for, routine production, and assess the impact of these extreme production parameters on the effectiveness of virus reduction steps.

When inactivation processes are validated, the rate of inactivation and the shape of the inactivation curves are assessed. For virus removal, the mass balance of virus infectivity in the starting material vs virus infectivity in the final sample and the final fractions has to be established.

Virus reduction or clearance is achieved during the manufacturing process by removal of viruses. This is accomplished by manufacturing steps designed to purify and concentrate the desired protein, dedicated virus filtration steps, and inactivation of viruses. The inactivation of viruses uses dedicated steps introduced into the manufacturing process.

## Virus Inactivation

### Pasteurization

Albumin solutions are heated as a liquid at 60°C for 10 to 11 hours in the final containers. Low concentrations of sodium

caprylate alone or with N-actyl tryptophan are added before sterile filtration and filling to prevent albumin denaturation. The safety of the method has been demonstrated for decades, although there is partition of virus during the ethanol fractionation that contributes to the removal of viruses and, therefore, to the overall virus safety.

It was known that most proteins denature when heated in solution to 60°C, a fact that explains the reluctance to apply this method to coagulation factors and other derivatives. But it is possible to pasteurize Factor VIII concentrates in the presence of high concentrations of glycine and sucrose or selected salts. This approach was developed by Heimburger in the late 1970s, resulting in a Factor VIII/vWF product with no proven cases of virus transmissions in more than 25 years. All parts of the tank must be heated equally to provide constant temperature in the aqueous solution. The impact of stabilizers and temperature on the virus inactivation capacity for a wide range of viruses must be validated. Data demonstrate the effective inactivation of a wide range of viruses of diverse physical and chemical characteristics. Enveloped and nonenveloped viruses are inactivated by pasteurization even in stabilized solution.<sup>17</sup>

### **Dry Heat**

After lyophilization of proteins (in the final container) to remove water, heating at 80°C for 72 hours or 100°C for 30 minutes is also an effective virus inactivation step. Residual moisture interferes with the success of this method; the inactivation of some viruses was greater at higher residual moisture but under such conditions the stability of the product was reduced. Thus, the residual moisture has to be controlled in each vial to remain between predefined limits demonstrated in validation studies.<sup>18</sup>

### **Vapor Heat**

Lyophilized (intermediate) products are heated at defined (relatively high) residual moisture—for eg, 60°C for 10 hours followed by 80°C for 1 hour. Low residual moisture levels of dry heat treatment do not interfere with the success of this method because the product intermediate is further processed to the final product.

### **Solvent/Detergent Treatment**

Organic SD treatment works by disrupting the lipid membrane of enveloped viruses, rendering them unable to bind to and infect cells. This method is not effective against nonenveloped viruses. An example of conditions used for virus inactivation is: 0.3% tri(n-butyl) phosphate (TNBP) and 1% ionic detergent (usually either Tween 80 or Triton X-100) at approximately 24°C for 4 hours (with Triton X-100) or 6 hours (with Tween 80). It is even possible to treat some preparations at 4°C. Product intermediate has to be filtered to eliminate virus trapped in gross aggregates. Stirring through the process is used to ensure homogeneity. Every droplet containing virus must be treated; solutions

are often transferred from one tank to another to ensure that material on the lid or surface of the first tank is subject to the treatment. At the end of the process, the SD mixture is removed by extraction with 5% vegetable oil, adsorption chromatography, or absorption on C-18 hydrophobic resin. This method has been very successful in inactivating HIV, HBV, and HCV. In virus validation studies, the key process variables such as concentration of the solvent and detergent compounds, the temperature, and incubation time as well as the impact of potential lipid content in the product intermediate (because this interferes with the availability of the SD compounds for virus inactivation), have to be covered.<sup>19</sup>

### **Low pH**

Immune globulins prepared at pH 4 will benefit from inactivation of many enveloped viruses. In general, the use of low pH cannot be relied upon and is adjunctive to other techniques.

### **Virus Removal by Methods for Protein Purification and Concentration**

Precipitation of proteins (ie, the precipitation of impurities or the protein of interest) results in the retention of the protein of interest or the impurities in solution. Most often the precipitate is removed or collected by depth filtration or centrifugation. Because the precipitation conditions are designed to purify/concentrate the protein of interest, these partitioning steps often remove only a small range of different viruses. Careful virus validation studies are required to assess the reliable contribution of such precipitation steps to the overall virus reduction for a final product.

### **Ethanol Precipitation**

Precipitation with ethanol (Fig 19-4) does have disinfectant properties, but minimally at the low temperatures typically used in plasma fractionation. Therefore, it provides little inactivation but it can partially separate virus from protein, leading to partitioning through precipitation. This was largely successful in ensuring the safety of immune globulin products along with the neutralization benefits of the antibody present.<sup>20</sup>

Comparable principles related to ethanol precipitation steps apply to other precipitation principles such as PEG precipitation, glycine and NaCl precipitation, or ammonium sulfate precipitation. Precipitation by octanoic acid adds another factor to precipitation steps, as octanoic acid inactivates enveloped viruses.

### **Chromatography**

Chromatography, such as ion exchange or hydrophobic interaction chromatography, can remove viruses, especially when the flow-through of a column is discarded (Fig 19-5). A further development of this concept is affinity chromatography whereby the protein of interest remains on the column even after washing and is eluted under defined conditions. Specially selected affinity chromatography (eg, monoclonal antibody

**Figure 19-4.** A diagram of virus removal by ethanol.

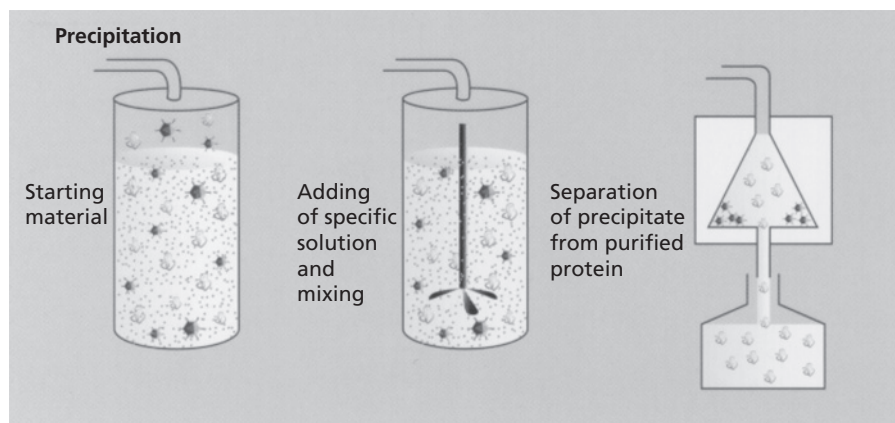
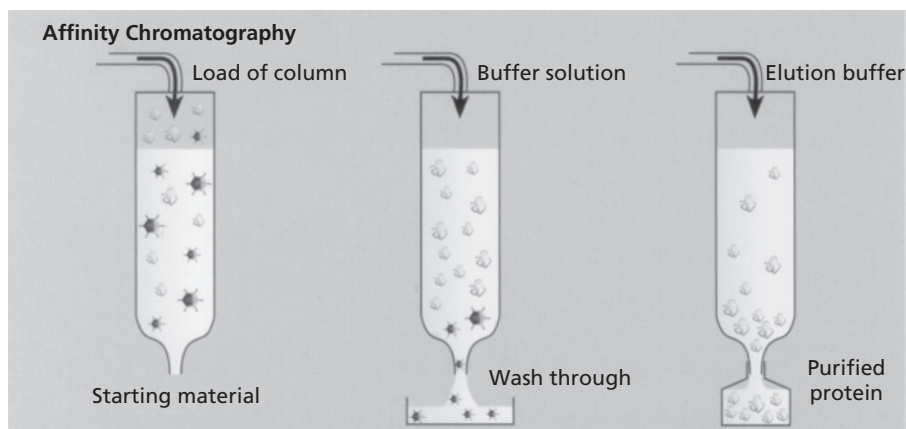


**Figure 19-5.** A diagram of virus removal by chromatography.



affinity chromatography for Factor VIII purification) results in a highly purified product from which much of the virus has been removed. When the resin of the chromatography column is reused, efficient sanitization of resins (and column parts) is a necessary step to avoid batch-to-batch contamination.

## Virus Removal by Dedicated Manufacturing Steps

### Virus Filtration

The measures of ethanol precipitation and chromatography explain why some derivatives have less viral contamination than others. Virus filtration, formerly called nanofiltration (Fig 19-6), is specifically designed to remove virus. It does so by removing virus based on size while allowing flow-through of the desired protein. Large protein molecules or easily aggregated proteins pose a challenge for this method. But virus filtration is a complex process. The success depends potentially not only on pore size but also on adsorption of the virus to the filter surface and removal of virus aggregated by inclusion in antigen-antibody or lipid complexes. Virus filtration is gentle, but appropriate testing must ensure that shear forces are not damaging the proteins.

Besides the established virus reduction factors discussed above, research into methods as irradiation and nucleic acid

intercalating substances is ongoing. The suitability of these inactivation methods with respect to modification of proteins has still to be demonstrated.

In order to achieve virus-safe plasma-derived products, effective virus reduction steps inherent in the manufacturing process are essential. Often two distinct effective virus reduction methods are implemented that complement each other in their mode of action. If for one manufacturing step a gap is demonstrated in the virus reduction capacity for a range of viruses, a second step covering that gap has to be introduced into the manufacturing process, eg, dry heat treatment or virus filtration for the reduction of nonenveloped viruses together with SD treatment. If one virus reduction step is effective for a wide range of viruses including enveloped and nonenveloped viruses, the manufacturing process of a plasma-derived product has to contain further steps which reliably (but not necessarily effectively) contribute to the overall virus reduction (according to the European guideline).

### Prions

Prions, the causative agents of human neurodegenerative diseases such as Creutzfeldt-Jakob disease (CJD) and variant CJD, cannot

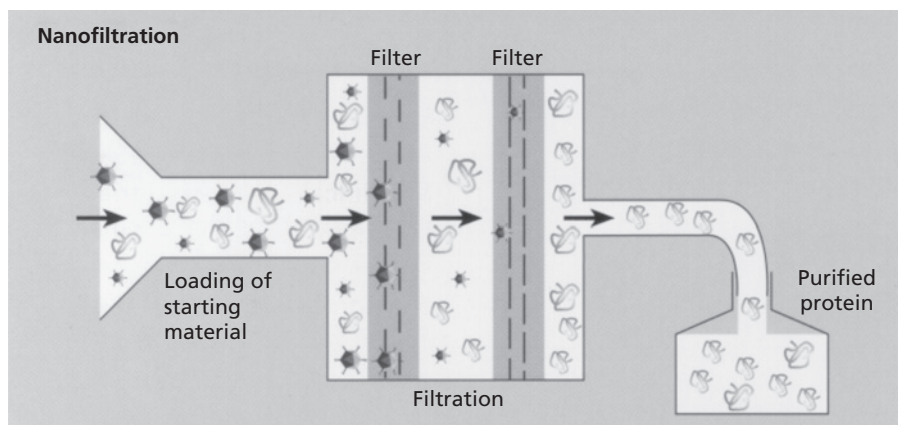


**Figure 19-6.** A diagram of virus removal by size exclusion.

be inactivated under production conditions for plasma-derived products. However, manufacturing processes of plasma-derived products are able to reduce transmissible spongiform encephalopathy infectivity, if it were in plasma, as assessed by the FDA and European regulatory agencies.<sup>21–23</sup>

### Assessment of Final Product

In general, it is not possible to test the final product for viral markers; such tests are not reliable and difficult to interpret. Also testing of final products by nucleic acid amplification testing (NAT) methods such as polymerase chain reaction is not suitable to demonstrate a safe product due to statistical methods of sampling (Poisson distribution); furthermore, NAT detects genomic sequences and not infectivity of a virus. This indicates the importance of the validation and the quality control of the manufacturing process.

The final product must be assessed to ensure virus reduction and intact product. Virus reduction is ensured by confirmation that the conditions validated for the process were rigidly adhered to and that opportunities for cross-contamination and recontamination have been prevented. This is particularly important in virus inactivation; there is less opportunity for this to occur with terminal virus inactivation such as the pasteurization of albumin. Regulatory authorities often require an assessment of virus safety. A probabilistic method is one that has recently been proposed and presented in some detail.<sup>24</sup>

Final product characterization is used to demonstrate that the therapeutic protein is present and intact. Total protein and pH assays are applied to all products, and tests for moisture and solubility are used for lyophilized products. Albumin is tested for protein composition and molecular size distribution; immune globulins are tested for IgG content, molecular size distribution, and potency of antibody reactivity; and in the case of intravenous and subcutaneous preparations, anti-complementary activity and clotting factor concentrates are tested for the activity of the specific clotting factor.

## Industry Safety Programs

### Regulatory Agencies

The major regulatory authorities for the global plasma industry are the FDA in the United States; the European Medicines Agency in Europe; the German Health Authorities and the Paul Ehrlich Institute in Germany; the Medicine and Healthcare Products Regulatory Agency in the UK; the Ministry of Health, Labor and Welfare in Japan; and the Therapeutic Goods Agency in Australia. Inspections must be carried out according to the national legislation as well as the Pharmaceutical Inspection Convention “GMP Guide for Blood Establishments for Whole Blood (Recovered Plasma)”<sup>25</sup> and “Guide to Inspections of Source Plasma Establishments and Plasma Warehouses”.<sup>26</sup>

### Voluntary Industry Standards

To assist with the regulatory and safety burdens, the Plasma Protein Therapeutics Association (PPTA), the industry trade group, has a certification program for collectors (International Quality Plasma Program or IQPP) and manufacturers (QSEAL). Table 19-5 shows the IQPP program and Table 19-6 shows the QSEAL program.

Donors must live in a set radius of the center (ie, not be transient or homeless). The PPTA in the United States maintains a national registry of donors who have been deferred for positive viral marker tests. That registry is checked for all new donors. Each donor is considered an applicant until the second donation is made and the test results from the first two donations are both acceptable. If the donor does not return, the unit is considered an “orphan” and cannot be used for injectable product. Viral marker results for the qualified donors (those who have passed applicant status) from a given center must be below a set level based on statistical analysis for the center to supply injectable product. Thus, the pillars of safety include: 1) selection including the donor questionnaire, collection activity, plasma testing for serology and NAT for HIV, HAV, HBV, HCV, and parvovirus B19, 2) the inventory hold to ensure no new information that

**Table 19-5.** IQPP Standards for Source Plasma<sup>27</sup>

- 
1. Qualified donor
  2. Community-based donor
  3. Use of National Donor Deferral Registry
  4. Education of donors at risk for infectious disease
  5. Viral marker standard
  6. Personnel education and training
  7. Professional medical facility criteria
  8. Quality assurance standard
- 

**Table 19-6.** Q-SEAL Standards for Fractionators<sup>27</sup>

- 
1. All plasma from qualified donors
  2. Viral marker standard from facilities
  3. 60-day inventory hold
  4. Nucleic acid amplification testing
  5. Consistency, quality, and traceability of intermediate products
  6. Parvovirus B19 testing
- 

would disqualify a donor emerges within 60 days after the donation, 3) look-back and traceability, 4) manufacturing including plasma pool testing, fractionation by GMP, virus inactivation and removal, batch-to-batch segregation, and virus validation studies, 5) quality control including final removal and batch release, and 6) monitoring including pharmacovigilance and traceability.

## Recombinant Deoxyribonucleic Acid Technology and the Manufacture of Plasma Derivatives

Genetic engineering has been used to isolate specific genes of human origin responsible for the production of distinct proteins. Transfer of these nucleic acid strands into cell culture systems and microorganisms has resulted in the economic production of the desired proteins. The derivatives produced by recombinant deoxyribonucleic acid (rDNA) technology that are in clinical use include Factor VIII, Factor IX, and Factor VIIa.

The process begins with a library of DNA sequences complementary to the messenger ribonucleic acid in a given tissue. Antigenic identification (using monoclonal antibodies raised against purified plasma protein) or hybridization (using oligonucleotide probes derived from amino acid sequences of the protein of interest) is used to create the specific complementary DNA.<sup>28</sup>

Currently two biologically active, full-length recombinant and one B-domain-restricted Factor VIII products are on the market after extensive clinical testing. More recently, B-domain-deleted recombinant Factor VIII has become available for clinical use.<sup>20</sup> In addition, a newly licensed recombinant Factor VIII grown in Chinese hamster ovary cells is free of any human or animal material. The in-vivo recovery and half-life are similar to those of plasma-derived Factor VIII.

The products are effective in preventing and treating hemorrhagic episodes in patients with hemophilia A.<sup>29,30</sup> The application of rDNA technology has led to the cloning and expression of other important plasma proteins, some of which (eg, Factor IX) are used routinely while others are still undergoing clinical evaluation.

## Summary and Outlook

The plasma industry exists in the interface between blood transfusion and pharmaceuticals. Some of its products could be replaced by recombinant products. The economics of the industry require fractionation of clotting factor concentrates from each liter. The products have become more costly, but the safety has been significantly enhanced. The cost has created challenges for patients, health insurance plans, and governments that purchase the products; thus, these products are much less available in developing countries.

There always remains the potential of finding a new therapeutic product in plasma. For example, a substance from plasma (apolipoprotein A-1) that increases HDL (good cholesterol) is now in clinical trials and could significantly affect the prevention of heart disease.<sup>31</sup>

Growth in the plasma industry worldwide is expected to continue as more products are brought to more patients. It is hoped that the major enhancement to public health seen with the near elimination of hemolytic disease of the fetus and newborn due to RhIG can be repeated for more and more patients in the years ahead.

## Acknowledgments

Tables 19-1, 19-3, and 19-4 and Fig 19-1 were reproduced from the chapter by W.G. Van Aken in the previous edition of this book. The authors of the current chapter appreciate the strong foundation provided in the earlier work.

## Disclaimer

The authors have disclosed no conflicts of interest.

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Plasma is the aqueous component of blood in which many different cellular elements and macromolecules are suspended, but it is the proteins that have been the focus of interest for transfusion medicine, including specifically albumin, coagulation factors, and immunoglobulins. This chapter discusses clinical use of the first two groups, with a main focus on levels of evidence for effectiveness of Fresh Frozen Plasma (FFP). Related material is found in Chapters 17 and 38 through 40.

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**Frozen Plasma and Fresh Frozen Plasma**

Plasma for transfusion can be obtained through centrifugation of whole blood or through the use of plasmapheresis. It can be stored frozen as whole plasma or used to produce more purified constituents, including concentrates of coagulation factors and fibrin sealant, immunoglobulins (normal or specific, eg, Rh Immune Globulin), anticoagulants (eg, antithrombin, protein C), complement-related proteins (C1-esterase inhibitor), and albumin.

Fresh Frozen Plasma is human donor plasma frozen within a short specified period after collection (often 8 hours) and then stored at a defined temperature, typically  $-30^{\circ}\text{C}$ . Plasma frozen at slightly later intervals (typically up to 24 hours) after collection is referred to as Frozen Plasma (FP) or Plasma Frozen within 24 Hours after Phlebotomy (FP24). Levels of the labile coagulation Factors V and VIII are slightly lower in FP in comparison to FFP, but both components are usually considered clinically equivalent. For the purposes of this chapter the two components are covered (interchangeably) using the common term FP, unless otherwise stated. However, the marginally lower levels for some

coagulation factors in FP24 may be clinically important—for example, in the context of transfusions to small neonates.

After thawing for transfusion, FP contains near normal levels of most plasma proteins, including procoagulant and inhibitory components of the coagulation system, acute phase proteins, immunoglobulins, and albumin, although all levels will be further diluted by citrate anticoagulant solution. Factor VIII is typically the only plasma protein whose level is quality-controlled to meet component specifications as required by guidelines in the United Kingdom (UK)<sup>1</sup> and European Union (EU) [but not in the United States by the Food and Drug Administration (FDA) or the AABB]; this threshold needs to be met for a proportion of units (typically 75%).

Coagulation factor content is well maintained in thawed FFP up to 5 days but with evidence of a decrease in Factor V and VIII. However, recommendations in many countries limit use to a defined shorter period after thawing. For example, current guidelines in the UK indicate that plasma may be used up to 24 hours after thawing. A typical unit of plasma derived from a collection of whole blood has a volume of just under 300 mL, and local and national guidelines for usage generally specify a dose of around 10 to 20 mL/kg.

The composition of FP can be influenced by many factors. These would include gender, age, and genetic, dietary, and other environmental factors, all potentially modifying levels of individual proteins. The composition of collected plasma would also be affected by the processing procedure, including how quickly the plasma is collected and then stored. Although clinicians tend to assume approximate equivalence in clinical effectiveness between individual units of FP, it is likely that there is heterogeneity. This variation reflects not only the biological differences in constituents between donors [eg, von Willebrand factor (vWF) and Factor VIII levels, being ABO dependent], but also differences in processing, storage, and preparation for administration. Such variation might be expected to be less marked for pooled plasma components such as solvent/detergent-treated FP.



Figure 20-1 shows data for procoagulant factor content from 66 FFP units.<sup>2,3</sup>

Which Patients Receive Frozen Plasma?

Clinical use of FP has grown steadily over the past two decades in many countries. Moreover, there is evidence of variation in usage both within and between countries. In a comparison of FP use in five countries, the ratio of FP units to Red Blood Cell (RBC) units transfused varied from 1:3.6 in the United States to 1:8.5 in France.<sup>4</sup> Two studies have reported a wide variation in the use of

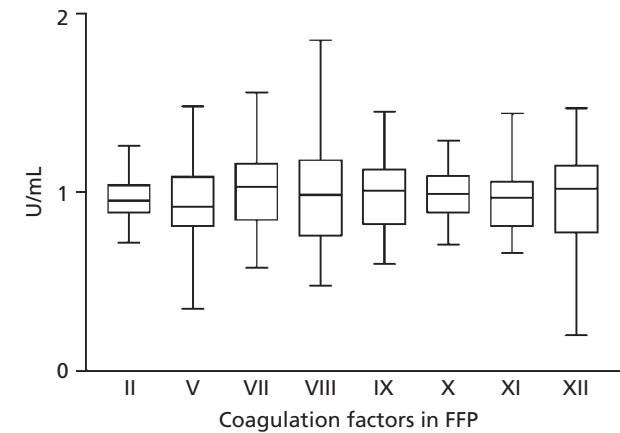


**Figure 20-1.** A “box-and-whisker” plot for the 25th and 75th percentiles, with median and error bars as range, for normal plasma (leukocyte-reduced FFP).

FP among facilities within the same country for patients undergoing cardiac surgery<sup>5,6</sup> and critical care patients.<sup>6</sup> In the latter study, lower use of FP in critical care patients did not correlate with lower use in cardiac surgery patients, which suggests that important variation in FP use within hospitals also exists.<sup>6</sup>

FP is given primarily for two indications: to prevent bleeding (prophylaxis) or stop bleeding (therapeutic). Prophylactic transfusions, which may account for over 50% of all FP transfusions, are largely given before surgery or an invasive procedure.<sup>7</sup> In a subset of Finnish hospitals, over 6000 FP units were tracked to 1159 transfused patients, revealing that FP was transfused most often to surgery patients, especially cardiac patients.<sup>8</sup> This is consistent with reports of FP transfusions in other countries.<sup>9-12</sup> Likewise, in the UK, data from an epidemiology study (EASTR) indicated that most FFP is used for patients with general medicine, liver disease/gastroenterology, and hematology conditions, as well as cardiothoracic, oral, general, and neurosurgery patients (C. Llewelyn, personal communication). Critical care patients also appear to make up the other large recipient group for FP transfusions<sup>10,12</sup> although this group may overlap with surgical patients in some surveys. Local audits in many countries additionally identify other recipients of FP as including patients requiring reversal of warfarin effect, or patients with disseminated intravascular coagulation (DIC), massive transfusion, or thrombotic thrombocytopenic purpura (TTP).

Further information on FP use in the UK, United States (US), and Australia is summarized in Tables 20-1 and 20-2. Table 20-1 describes studies that reported on the indications for FP

**Table 20-1.** Reported Reasons for Frozen Plasma Transfusions (Percentage of Transfusion Episodes)

| Indication for Frozen Plasma | Studies            |                    |                     |                   |                         |                         |
|------------------------------|--------------------|--------------------|---------------------|-------------------|-------------------------|-------------------------|
|                              | Hui <sup>12</sup>  | Dzik <sup>7*</sup> | Tobin <sup>10</sup> | Metz <sup>9</sup> | Tuckfield <sup>13</sup> | Shanberge <sup>11</sup> |
| Therapeutic                  | —                  | 35                 | —                   | —                 | —                       | 89                      |
| Prophylactic                 | —                  | 48                 | —                   | —                 | —                       | 11                      |
| Bleeding                     | —                  | —                  | 27 <sup>†</sup>     | 27 <sup>†</sup>   | 31 <sup>†</sup>         | —                       |
| Perioperative                | —                  | —                  | 22 <sup>†</sup>     | 41 <sup>†</sup>   | 47 <sup>†</sup>         | —                       |
| Coagulopathy                 | 13                 | 8                  | —                   | 4                 | 10                      | —                       |
| Liver disease                | 13.5 <sup>‡</sup>  | —                  | 8.5 <sup>§</sup>    | —                 | —                       | 14.4                    |
| Warfarin                     | 32.5 <sup>  </sup> | 21                 | 36.5                | 11.5              | 4                       | 24.0                    |
| Cardiac surgery              | 6.5 <sup>‡</sup>   | —                  | —                   | —                 | —                       | 16.5                    |
| AAA                          | —                  | —                  | —                   | —                 | —                       | 7.0                     |
| Massive transfusion          | 24 <sup>‡</sup>    | —                  | —                   | 16 <sup>‡</sup>   | 7.5 <sup>‡</sup>        | 2.1                     |
| ICU coagulopathy             | —                  | —                  | —                   | —                 | —                       | 3.9                     |
| DIC                          | 8.5 <sup>‡</sup>   | —                  | —                   | —                 | —                       | —                       |
| Other                        | —                  | 8                  | —                   | —                 | —                       | 23.6                    |

\* does not include operating room requests

† correct coagulopathy

‡ with bleeding

§ prophylactic, associated with warfarin or liver disease

|| immediate reversal required

AAA = abdominal aortic aneurysm; ICU = intensive care unit; DIC = disseminated intravascular coagulation

transfusions. Table 20-2 contains data on the use of FP by medical/surgical specialties.

### The Side Effects of Frozen Plasma

Crucial to recommendations in guidelines for FFP transfusion practice is the need for a clear understanding of the risk of harm. FP is not without risk, and indeed may be among the most “high risk” of all blood components.<sup>14,15</sup> The most immediate serious complication of FP is transfusion-related acute lung injury (TRALI), although there are ongoing issues of reporting and diagnosis of this condition, which make accurate estimation of prevalence difficult.<sup>16,17</sup> Other risks are transfusion-transmitted infection, including an unquantifiable risk of prion disease, and fluid overload, which may be a greater issue if larger doses of FP are transfused to attempt (full) reversal of abnormal coagulation tests (discussed later). These risks related to circulatory overload are poorly understood, but could be important for critically ill patients, for example those in the intensive care unit (ICU). Allergic reactions to FP are relatively common, with a frequency of around 1% to 3% of all transfusions, and can be extremely troublesome, or rarely, life threatening, for some multitransfused patients. In the UK, an association between cases of TRALI and female donors has been identified through the Serious Hazards of Transfusion hemovigilance system and male donors are now used as much as possible for production of FFP.<sup>18</sup> In the United States, the AABB has recommended that blood collecting facilities should implement interventions to minimize the preparation of high plasma-volume components from donors known to be leukocyte-alloimmunized or at increased risk of leukocyte alloimmunization.<sup>19</sup>

Understanding the risks of FP transfusion is important when physicians consider the use of FP as prophylaxis, which as stated is a common indication for FP in clinical practice. A prophylactic policy should be justified only if the risk of bleeding is greater than the risk of harmful effects. Without evidence of benefit, such a policy aimed at preventing (uncommon) bleeding

complications could involve transfusing (harmful) FP to a large number of patients, many of whom might not bleed even if prophylactic FP were not given.

### Summary of Recommendations from Guidelines for the Use of Frozen Plasma

Recommendations for the transfusion of FP have remained relatively consistent over the years for the transfusion of FFP, including: 1) active bleeding, or before surgery or an invasive procedure in patients (adults and neonates) with acquired deficiencies of one or more coagulation factors as demonstrated by an increased international normalized ratio (INR), prothrombin time (PT), or activated partial thromboplastin time (aPTT), when no alternative therapies are available or appropriate; 2) immediate correction of vitamin K deficiency or reversal of warfarin effect in a patient with active bleeding, or before surgery or an invasive procedure (in conjunction with use of prothrombin complex concentrates); 3) DIC or consumptive coagulopathy with active bleeding; 4) TTP; and 5) active bleeding, or before surgery or an invasive procedure in patients with a congenital factor deficiency when no alternative therapies are available or appropriate. Variations of these indications will be found in most national guidelines.<sup>20-25</sup>

Previous common uses of FFP that are now considered inappropriate include: volume replacement, correction of hypoalbuminemia, nutritional support, and immunoglobulin replacement.

### Evidence for Indications

It is important to identify whether the *presumed* benefits of FP really outweigh the *real* risks. The clearest evidence for a direct beneficial effect of FP would be expected to come from randomized controlled trials (RCTs) of FP compared with no FP/plasma. Studies of interventions comparing FP with a non-blood product (eg, solutions of colloids and/or crystalloids) may also assess effectiveness, but these studies would need to be separately evaluated given that these solutions have variable effects on in-vivo or in-vitro coagulation.<sup>26</sup>

Of a total of 57 RCTs published through 2004 on the use of FP, only 17 compared FP to no FP or a colloid/crystalloid solution in adults.<sup>27</sup> Many studies enrolled small numbers of patients, and provided inadequate information on the ability of the trial to detect, without bias, meaningful differences in outcomes between the two patient groups. When considering all RCTs evaluating prophylactic usage across a range of settings (including cardiac, neonatal, and other clinical conditions) as a group, the overall findings failed to document evidence for the effectiveness of prophylactic FP, for a range of clinical and laboratory outcomes.

In two large, well-conducted trials,<sup>28,29</sup> evidence for a lack of benefit for prophylactic use of FP was reported. Both studies were designed to evaluate carefully the effectiveness of FP in a large group of patients, and provided information about the sample sizes required to allow adequate power to detect clinically important differences between the groups of patients. In

**Table 20-2.** Reported Frozen Plasma Transfusions by Medical/Surgical Specialty (Percentage of Transfusion Episodes)

| Medical Specialty     | Studies           |                         |                     |                   |
|-----------------------|-------------------|-------------------------|---------------------|-------------------|
|                       | Hui <sup>12</sup> | Shanberge <sup>11</sup> | Tobin <sup>10</sup> | Metz <sup>9</sup> |
| Medical               | 13                | 48.8                    | 27.5                | 35                |
| Oncology              | —                 | —                       | 3                   | 8                 |
| Surgical              | 36                | 46                      | 24.5*               | 31*               |
| Cardiac surgery       | 6                 | —                       | 8.5                 | 23                |
| Orthopedics           | —                 | —                       | 3.0                 | 2                 |
| Obstetrics/gynecology | —                 | 1.8                     | —                   | 1                 |
| Critical care         | 39                | —                       | 39.5                | —                 |
| Emergency             | 14                | —                       | 5                   | —                 |
| Pediatrics            | —                 | 3.1                     | —                   | —                 |

\* Not including cardiac and orthopedic surgery

the first trial, the Northern Neonatal Nursing Initiative (NNNI) Trial Group randomly assigned 776 neonates to receive FP or volume expanders (gelofusin or dextrose-saline); results did not show any differences in the prevention of intraventricular hemorrhage.<sup>28</sup> Allocation concealment and blinding of outcome assessors (to monitor clinically relevant long-term developmental outcomes) was reported. Of note, the study did not include measurement of coagulation tests. In the other trial, the effectiveness of FP was evaluated in patients with acute pancreatitis.<sup>29</sup> In total, 275 patients were randomly assigned to receive either FP or a colloid solution. No evidence for benefit was reported.

In summary, this review of the RCT literature raises important questions about current levels of evidence to support use of FP in clinical practice, including in particular prophylactic use where evidence for a beneficial effect is weak. Specific information from RCTs and observational studies about additional selected clinical groups is discussed below.

### Cardiac Surgery

Epidemiologic evidence indicates that FP given before or during cardiac surgery accounts for approximately 5% to 10% of all use,<sup>10,12</sup> and a number of published RCTs have assessed benefit. However, RCTs comparing prophylactic use of FP to either no FP or a non-plasma product after cardiopulmonary bypass have not shown evidence of a consistent significant beneficial effect on blood loss or transfusion requirements.<sup>27</sup> A meta-analysis of the results from these trials has also failed to establish evidence for any benefit.<sup>30</sup> The hemostatic changes related to cardiac bypass are multifactorial, and not solely related to coagulation factor deficiency; thus, it is not surprising that prophylactic FP use fails to provide benefit.<sup>31</sup> Treatment of bleeding associated with abnormalities of coagulation tests after surgery should be treated with FP in the same manner as DIC.

### Massive Transfusion, Trauma, and Disseminated Intravascular Coagulation

Very few trials have evaluated the effects of therapeutic FP in patients with bleeding who have multiple or global deficiencies of coagulation factors (eg, DIC or massive transfusion), presumably in part reflecting the difficulties of trial design in this setting. From a pathophysiologic perspective, use of FP in this setting seems clinically appropriate. One study tried to address the effectiveness of FP use in DIC in a group of neonates, using defined criteria for a diagnosis of DIC.<sup>32</sup> In this small three-way controlled trial, neonates were randomly assigned to receive 1) exchange transfusion using whole blood, 2) FP and platelet infusions, or 3) no plasma (control group). Although there were no differences in rates of improvement for coagulation tests or in survival in either treatment group, the size of the trial was small (a total of 33 patients across three arms).

Bleeding in trauma patients is typically multifactorial, but there is now increasing evidence that early “coagulopathy” is a significant factor associated with poor outcome. Although guidelines for FP transfusion in this scenario frequently support use of

plasma when coagulation tests are documented to be abnormal, there is a risk that this reactive response, coupled with slow turnaround time for obtaining laboratory results and thawing plasma for transfusion, can lead to the development of marked disturbances of coagulation. This might be an example of the risk of undertransfusion of FP, in contrast to much of the emphasis in this chapter on the risks of inappropriate overtransfusion. There is interest in defining the optimal transfusion support for massive transfusion, including the evaluation of the early use of FP in trauma patients.<sup>33</sup> A number of retrospective studies point to improvements in survival for patients with trauma when red cell:plasma ratios of transfusion support are nearer to 1:1.<sup>34</sup> Strategies describing more aggressive use of FFP and platelets have also been reported recently in surgical practice.<sup>35</sup>

### Intensive Care

In the ICU setting, few prospective data exist on the frequency with which FP is given as prophylaxis—eg, before central venous cannulation or other invasive procedures. The use of FP before central venous cannulation is debatable; the evidence would suggest that the rate of important hemorrhagic complications associated with central venous cannulation is very low even in patients with normal or abnormal coagulation test results.<sup>36-39</sup>

Dara et al<sup>40</sup> reported a single-center retrospective cohort study of FP use in medical ICU patients. They identified patients in whom an INR  $\geq 1.5$  was found during ICU stay and evaluated FP use in the subgroup of patients who were not actively bleeding. In addition to variability in FP transfusion practice, the authors observed that patients who received FP had a similar rate of hemorrhage to matched cases, but had a higher incidence of “acute lung injury” during the 48 hours after transfusion (18% vs 4%;  $p = 0.02$ ). This association raises the possibility that critically ill patients, many with concurrent inflammatory problems, may be more susceptible to TRALI after receiving plasma-rich blood components, although distinguishing TRALI from other clinical problems such as volume overload remains problematic. These findings do not prove cause and effect, but again emphasize a need for concern about inappropriate use of FP, in view of the real risk of harm.<sup>16,41</sup>

### Liver Disease

The coagulopathy of liver disease is complex, with abnormalities of platelets, fibrinolysis, and inhibitors of coagulation as well as coagulation factor deficiency. Where possible, deficiency of vitamin K should always be corrected first before considering the role of plasma transfusion. One early RCT<sup>42</sup> attempted to assess the effects of regular prophylactic transfusions of FP in patients with paracetamol overdose. Twenty patients were evaluated and no effects on bleeding, morbidity, and mortality were observed. However, the small size of the study and the crossover design were not optimal for detecting differences between the two groups.

Other studies to investigate FP transfusion practice in patients with liver disease have been uncontrolled and observational.

Youssef et al<sup>43</sup> reported the effects of FP transfusion in 100 patients with liver disease, and found that it was difficult to correct abnormalities of coagulation screening tests unless large volumes of FP were transfused, and that the effects of transfusion were short-lived. Lack of evidence for an association between bleeding and laboratory tests of coagulation in liver disease has also been reported in a number of studies (eg, the retrospective studies of McVay and Toy<sup>44</sup>). Although these studies contain no control group data, there is again a consistent theme of lack of evidence for benefit for FP transfusion in patients with liver disease. Of note, the lack of bleeding in cirrhotic patients despite diminished procoagulant synthesis (and abnormal PT/aPTT) may be explained by a parallel reduction in the production of anticoagulant proteins, such as proteins C and S, leading to equivalent thrombin generation potential on activation of both pro- and anticoagulant pathways.<sup>45</sup>

In summary, guidelines for plasma transfusion typically include the multiple coagulation factor deficiency state of liver disease associated with bleeding or before invasive procedures. However, the number of relevant studies published to support these statements is very limited and it is far from clear just how effective this transfusion policy is. In particular, observational studies evaluating common invasive procedures such as liver biopsy, paracentesis, or central venous cannulation indicate that moderate disturbances of coagulation tests are, in fact, not predictive for bleeding risk.<sup>46,47</sup> Large volumes of FFP appear to be needed to correct abnormalities of coagulation tests, and the effect is short-lived. In addition, as mentioned later, although the PT or INR (and aPTT) are widely employed for patients with liver disease, their clinical validity in this patient group is unproven.

### Reversal of Warfarin Effect

In addition to vitamin K, guidelines recommend FP or prothrombin complex concentrates (PCCs) for reversal of excessive anticoagulation but only in patients with major bleeding.<sup>48</sup> However, there is continuing controversy over which component is preferable; this, in part, reflects a lack of clinical trials directly comparing the two components. PCCs are produced by fractionation of pooled plasma and contain coagulation Factors II, IX, and X at a significantly higher concentration than FP. But not all PCCs are the same and Factor VII levels do vary. In a retrospective single-institution study (n = 41), Makris et al<sup>49</sup> reported that patients receiving PCCs achieved more rapid and complete reversal of abnormalities of coagulation testing than patients receiving FFP. However, the results of this study should be interpreted with some caution because significant differences in baseline coagulation test results existed in the two groups and the authors did not report any bleeding outcomes. Further evaluation is required to ascertain whether the more rapid improvements in coagulation screening tests translate into clinical benefit, without an increased risk of thromboembolic complications. In the absence of major bleeding associated with excessive anticoagulation due to vitamin K antagonists, primary treatment should be initiated with oral or intravenous vitamin K.

### Therapeutic Apheresis—Thrombotic Thrombocytopenic Purpura

FP is frequently used as a replacement fluid in patients undergoing therapeutic apheresis procedures.<sup>50</sup> On the basis of findings from one RCT,<sup>51</sup> plasma exchange with FP is first-line treatment for TTP;<sup>52</sup> the FP providing a source of ADAMTS13. However, definitive randomized studies to define the optimal dose and schedule of FP or type of plasma (eg, cryosupernatant, solvent/detergent-treated) for therapeutic apheresis in patients with TTP have not been undertaken. Of interest, early studies investigating plasma exchange for conditions other than TTP have reported that despite repeated procedures with replacement fluids not containing coagulation factors, no bleeding complications occurred, even though marked reductions of coagulation factor levels were seen.<sup>53</sup>

### Is There an Optimal Dose of Frozen Plasma?

As described in the section on patients with liver disease, there are important questions about the optimal dose of FP, and not infrequently only large volumes achieve correction of coagulation test results. However, questions about appropriate or optimal dose for FP transfusion generally presuppose that evidence of dose-dependent effectiveness exists in the first place. Much of the evidence concerning appropriate dose comes from a derived synthesis of physiologic assessments of coagulation factor content and effects of plasma infusion.<sup>54</sup>

There is evidence that FP may be ineffective in correcting mild-to-moderate abnormalities of coagulation screen tests. Abdel-Wahab et al<sup>55</sup> prospectively evaluated the effects of plasma transfusions on PT/INR in hospital patients with prolonged INRs. They followed patients with a pretransfusion PT between 13.1 seconds and 17 seconds (INR equivalent 1.1-1.85). A total of 324 plasma units were evaluated in 121 patients. Fewer than 1% of patients had normalization of PT/INR following transfusion, and only 15% demonstrated a correction of halfway to normal. Of interest, there was also no meaningful correlation with clinical bleeding, as recorded retrospectively by patient chart review. Finally, when all cases of transfusion were reviewed for correction, there was little evidence for dose-response effect.

In another study,<sup>56</sup> 22 critically ill adult patients were allocated in consecutive groups to receive a lower dose of FP (median volume, 12.2 mL/kg) or a higher dose (median volume, 33.5 mL/kg) with the aim to increase hemostatic coagulation factor levels to >30 IU/dL (based on experience of treating patients with hemophilia). The lower dose increased the coagulation factor levels above the desired threshold in only one of five patients; with the higher dose, all seven patients achieved the target levels. Many patients who received the lower (standard) dose, but not larger dose, of FP failed to achieve the target level of coagulation factor replacement. Another study<sup>57</sup> reported on 103 adult patients with minimally prolonged INRs, and found that adding FP to their treatment failed to change the decrease in INR that occurred over time. When other studies or trials do report apparent “correction,” the overall absolute or mean changes again

appear very small. For example, in one RCT of patients with liver disease, the median reduction in INR attained after FFP was 0.2 (range 0–0.7).<sup>58</sup>

In summary, although the initial dose should be based on patient weight (typically 10–20 mL/kg), an optimal dose for clinical use has not been validated or defined in larger trials. The effectiveness of different doses may also be limited by clinical situation—eg, rapid ongoing consumption of coagulation factors in DIC, or risks of circulatory overload if larger doses are given with the intention of achieving full correction of coagulation screen tests.<sup>17</sup>

It should be appreciated that the outcomes for many studies of plasma, including dose, are laboratory-based, particularly centered around the PT/aPTT. But correlations between these coagulation screen tests and coagulation factor levels are poor and nonlinear, with limitations in expectations of achieving correction, particularly as factor levels approach normal. The laboratory tests of aPTT and PT were developed to investigate coagulation factor deficiencies in patients with a bleeding history by providing an end-assessment of thrombin generation by fibrin formation; therefore, the important issue of their applied clinical validity continues to be raised. The PT and aPTT results may be abnormal for a number of reasons not associated with bleeding risk (eg, the normal variation between individuals).<sup>59</sup> Some laboratories report the INR, and physicians then base decisions to transfuse plasma on results above a certain threshold, typically 1.5 times the control. But again, the INR is based on PT and was developed to monitor warfarin therapy, by standardizing results to account for different sensitivities of thromboplastins. The extrapolation of PT to INR may really be valid only for those patients stably anticoagulated with vitamin K antagonists.<sup>60</sup>

Finally, an important study by Deitcher<sup>61</sup> showed that over the INR range of 1.3 to 1.9 inclusive, mean factor levels ranged from 31% to 65% (Factor II), 40% to 70% (Factor V), and 22% to 60% (Factor VII). All of these levels are consistent with adequate concentrations of factors to support hemostasis. These results would be consistent with the findings from observational studies mentioned earlier, pointing to a lack of predictive effect for bleeding risk in patients with mild/moderate abnormalities of coagulation tests.<sup>47</sup>

### Clinical Effectiveness of Pathogen-Inactivated Plasma

Pathogen reduction has been applied to plasma to reduce the risk of transfusion-transmitted infection. The main methods are methylene-blue treatment, in which single-donor units are treated with methylene blue and light; solvent/detergent treatment in which several thousand plasma donations are pooled before exposure to a solvent and detergent; and amotosalen treatment in which a psoralen (S-59) is added to plasma before exposure to ultraviolet A light. Of these, only solvent/detergent treatment has been licensed by the FDA, and none of these methods are currently widely available in the United States. Although the methods offer good virus protection and can reduce microbial infectivity, it should be noted that these

processes are associated with alteration or loss of coagulation factor content.<sup>62,63</sup> There have also been some suggestions that solvent/detergent-treated FFP may be associated with reduced risk of TRALI, although this has not been confirmed.

Pathogen-inactivated formulations will probably increasingly find their way into clinical practice. In the UK, plasma is now imported from the United States for children and neonates up to the age of 16 because of perceived lower risk of variant Creutzfeldt-Jakob disease transmission. It is pathogen inactivated (methylene-blue treated) because there is a higher background level of viral markers in the US population compared to the UK.

Studies of efficacy comparing pathogen-inactivated products with standard FP have different aims than trials in which FP is compared to no treatment or a placebo.<sup>64</sup> Arguably, assessing the clinical efficacy of these pathogen-inactivated variants of FP is very difficult when evidence of benefit for the standard product is thin, which broadly is the case for use of plasma across many clinical settings. Although newer pathogen-inactivation processes are aimed at reducing the real but low risk of infection transmission, they may paradoxically have altered clinical “effectiveness”—at the cost of marginally increased safety. Solvent/detergent-treated plasma is known to have lower levels of some inhibitors of coagulation (eg, protein S) and there is one case report suggesting an increased incidence of thrombosis in patients with TTP receiving this product compared to standard FP.<sup>65</sup> Transfusion of pathogen-inactivated FP has also been associated with a need for a greater volume in at least one retrospective study,<sup>66</sup> although the reasons for this are unclear.

### Clinical Effectiveness of Cryoprecipitate

Cryoprecipitate represented the first practical preparation of a concentrated form of antihemophilic factor. It is prepared by controlled thawing of frozen plasma to precipitate high-molecular-weight proteins, including Factor VIII, vWF, and fibrinogen. In many developed countries, cryoprecipitate is transfused mainly as a concentrated source of fibrinogen. An adult dose of around 10 single-donor units of cryoprecipitate typically raises the plasma fibrinogen level by up to 1 g/L. The remaining product, following the removal of cryoprecipitate, is termed cryosupernatant (cryoprecipitate-reduced plasma). It has been used in the treatment of TTP, because of the theoretical benefit of its reduced content of vWF high-molecular-weight multimers, although the added benefit of cryosupernatant has not been proven.<sup>67</sup>

However, FP contains fibrinogen at near normal plasma levels, and so will correct low fibrinogen levels in many situations if the volumes for infusion are adequate. Therefore, cryoprecipitate should not be considered for transfusion solely as a more concentrated form of FP (eg, when there are concerns about fluid overload), because it predominantly contains only Factor VIII, vWF, fibronectin, Factor XIII, and fibrinogen. In other words, cryoprecipitate is not a source of all coagulation factors and, therefore, is not appropriate replacement therapy in patients with global coagulation factor deficiencies (eg, with liver disease). Its use should be reserved for patients with documented isolated

hypofibrinogenemia. There are few prospective trial data to define optimal use of cryoprecipitate. There is anecdotal evidence that the use of cryoprecipitate is rising in many countries, although the exact reasons for this remain unclear. Finally, a specific purified fibrinogen concentrate is now available, and it may represent a safer concentrate for direct fibrinogen replacement in isolated deficiencies (eg, inherited hypofibrinogenemia). However, this product is not licensed for clinical use in many countries.

### Use of Guidelines for Plasma Transfusion

Although practice guidelines produced by national and professional organizations for FP transfusion are readily available in many countries, there continues to be evidence for variation in practice.<sup>5-7</sup> The result is that FP appears to have the highest rate of inappropriate use (up to 50%<sup>68</sup>) among standard blood components with evidence of inappropriate practice derived from local audits. Table 20-3 summarizes results from a number of studies or audits that evaluate the appropriateness of transfusion of FP, according to local or national guidelines.

The reasons for poor compliance with guidelines are not entirely clear. Poor compliance may reflect misunderstandings about the limitations of coagulation tests; it may also reflect the difficulties in defining appropriate use in the face of a limited evidence base; and it may reflect that for many physicians, transfusion medicine practice is passed down from mentors who used blood components in a more liberal way. Perhaps now a more systematic approach is required to understand the determinants of this prescribing pattern and barriers to practice change.<sup>75,76</sup> This would include identifying better strategies for delivery and uptake of evidence-based health care, which are clearly demonstrated as unpredictable for FP.

No one would doubt the need to optimize FP transfusion practice; indeed, the best way to reduce the risk of adverse reactions is to define who really needs FP in the first place, and to reduce unnecessary transfusions. Unfortunately, even fewer data exist on the relative or absolute effectiveness of the different strategies or interventions that can be used to change transfusion prescribing behavior (eg, audit feedback, guidelines, educational sessions). A recent systematic review of the RCT evidence for the effectiveness of different educational strategies operating in transfusion medicine has pointed out the very weakness of the evidence itself for the success of these approaches to deliver sustained and effective behavioral change.<sup>77,78</sup>

### Conclusions

The recommendations for FP transfusion stated earlier form the basis for clinical indications for plasma. These specific limited indications should form the basis for local guidelines, which should help to minimize inappropriate use (eg, as prophylaxis in the absence of bleeding with mild to moderate abnormalities of coagulation tests or as volume expanders). Therapeutic uses of FP, in association with abnormalities of coagulation tests (except in emergencies) are based on a pathophysiologic rationale, but questions about optimal dose and schedule remain unanswered. A recurring theme in many settings is an overall paucity of high-level evidence to inform optimal FP transfusion practice, and there is a pressing need to undertake new trials of efficacy of FP transfusion. This will not only define which groups of patients really benefit from FP, but also identify the constituents in plasma that are providing the most benefit (eg, by analogy the role of ADAMTS13 in TTP). New studies need to take account of the extent to which adverse effects might negate the benefits of treatment with FP, including the unknown risks related to circulatory overload and TRALI.

**Table 20-3.** Studies Evaluating Appropriateness of Frozen Plasma Transfusions

| Study                          | Period of Study | No. of Patients | No. of Frozen Plasma Transfusions | No. of Units | Percentage of Inappropriate Transfusions (Source of Guidelines) |
|--------------------------------|-----------------|-----------------|-----------------------------------|--------------|-----------------------------------------------------------------|
| Hui et al <sup>12</sup>        | 2002-03         | NR              | NR                                | 793          | 10% (National)                                                  |
| Scholfield et al <sup>69</sup> | 2000            | NR              | NR                                | 669          | 37% (National)                                                  |
| Tobin et al <sup>10</sup>      | 1999            | NR              | 100                               | 411          | 57% (National)                                                  |
| Luk et al <sup>68</sup>        | 1999            | 358             | 671                               | 2372         | 53% (National)                                                  |
| Jones et al <sup>70</sup>      | 1996            | 41              | NR                                | 216          | 34% (National)                                                  |
| Metz et al <sup>9</sup>        | 1993-94         | NR              | 200                               | 944          | 31% (National)                                                  |
| Cheng et al <sup>71</sup>      | 1994            | NR              | 1152                              | 5635         | 43% (Local)                                                     |
| Shanberge et al <sup>11</sup>  | 1985-89         | 873             | NR                                | 2612         | 59% (Local)                                                     |
| Brien et al <sup>72</sup>      | 1986-87         | 83              | 106                               | 405          | 10% (Local)                                                     |
| Blumberg et al <sup>73</sup>   | 1981            | 135             | 160                               | 534          | NR                                                              |
| Silbert et al <sup>74</sup>    | 1979            | 102             | NR                                | 557          | NR                                                              |

NR = not reported.

In the future, with a better understanding of disorders with acquired deficiencies of one or more coagulation factors, replacement might be more appropriately managed with concentrates of multiple coagulation factors, which would minimize risks of circulatory overload. There should also be more open recognition of the limitations of current standard coagulation screening tests, and appreciation that in-vitro abnormalities of coagulation screening tests do not equate with an in-vivo failure of clinical hemostasis and the presence of clinical coagulopathy. Newer global tests of hemostasis may be better markers of abnormal bleeding risk, but should be assessed and validated in larger clinical studies.

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## Albumin

The optimal fluid regimen for correction of fluid and plasma volume disturbances in critically ill patients continues to be an area of controversy. The relevant fluids that may be infused as expanders include crystalloids (aqueous electrolyte solutions) or colloids (whether semisynthetic colloid solutions including gelatins, starches, and dextrans, or solutions of human albumin). This section of the chapter focuses on solutions of human albumin as a volume expander; synthetic colloid solutions and crystalloids are covered in other chapters.

Albumin is a very negatively charged protein produced in the liver and is the largest protein component of human serum. Its general functions include maintenance of osmotic pressure, transport of lipid-soluble substances, and a range of other biochemical effects such as buffering. Serum levels of albumin reflect changes as a consequence of either excessive losses (such as through the kidneys) or reduced production within the liver. As a broad generalization, low albumin levels have been associated in many patient groups with poorer outcomes.

Human albumin for transfusion is derived from plasma collected either by whole blood donation or by apheresis. As for any other pooled component, the concern is that a single infected donor unit can transmit disease to a large number of recipients. Therefore, multiple steps (eg, pasteurization) are added to the manufacturing process to limit the risk of infection transmission. In the UK, solutions of human albumin are available at protein concentrations of either 4.5% or 20%. Both solutions also contain sodium, other plasma proteins, and a stabilizer. Instructions for administration include risks of fluid overload, particularly for the higher concentration products, reflecting in turn the potential for rapid increases in colloidal osmotic pressure and intravascular volume, in addition to risks of sodium overload.

In general, hypoalbuminemia itself should not be considered an indication for albumin infusions, but when associated with fluid shifts and clinical consequences, infusions of human albumin may be indicated. Examples might include cirrhotic patients with refractory ascites who require serial therapeutic paracenteses, or patients with nephrotic syndrome associated with severe peripheral or pulmonary edema where diuretic therapy appears

ineffective. A solution of 4.5% human albumin is often the preferred choice in therapeutic apheresis, because it is iso-oncotic with plasma, susceptible to pasteurization or virus inactivation, convenient to administer, and associated with few adverse reactions. Its use is associated with a transient decline in the levels of all normal plasma constituents except albumin; only rarely is this of clinical significance. Patients with burns are at risk for multiple component deficiencies including secondary hypogammaglobulinemia, consumptive coagulopathy, and hypoalbuminemia; replacement requirements with albumin solutions can be significant in patients with extensive burns.

Although there are theoretical reasons why colloids including albumin may be more efficacious than crystalloids for 1) maintaining and expanding intravascular volume during resuscitation and 2) volume expansion in critically ill patients, these uses have been the subject of ongoing controversy, particularly in the light of systematic reviews and meta-analyses. An earlier systematic review by Schierhout and Roberts in 1998<sup>79</sup> started the controversy because it not only failed to find any benefit associated with colloids and human albumin solutions, but also raised concerns of harm—in this case, more deaths with the use of human albumin solutions compared to crystalloids. Despite some concerns about the methods of the meta-analysis (see other reviews<sup>80</sup>), this review not only raised awareness of meta-analysis as a tool for investigating evidence for clinical effectiveness, but crucially it gave impetus for subsequent definitive trials in the field of fluid resuscitation.

In particular, it was a driver for the recent completion of the Saline vs Albumin Fluid Evaluation (SAFE) study, the only large RCT (7000 participants) so far comparing the effect of fluid type (albumin solution vs normal saline) on clinical outcomes in hospital patients.<sup>81</sup> The SAFE trial found no significant differences in mortality for patients receiving 4.5% albumin or saline for fluid resuscitation. However, some of the interesting subgroup analyses—in patients with trauma, severe sepsis, and acute respiratory distress where differences were reported—really require further study to evaluate differences, as, the SAFE study was not a priori designed to answer questions about albumin use in these selected groups of patients. This need to think about subgroups and individual decisions may be important in these other clinical settings, until the evidence becomes available clinically. For example, older patients with heart disease might not tolerate infusions of saline alone with the attendant risks of circulatory overload; therefore, a mixture with albumin might be more appropriate.

Although the SAFE study appeared to resolve some previous safety concerns about albumin use and increased mortality, it is interesting to speculate on how albumin use has changed since the results of that study were published. On one hand there is some reassurance regarding the safety of albumin use, but on the other hand there remain uncertainties about cost-effectiveness, particularly by comparison to less expensive alternatives and colloids. In addition, clinical outcomes other than mortality were not a focus in the SAFE trial but remain important in the minds

of many clinicians who advocate albumin usage. In conclusion, although in many ways the SAFE study has created more uncertainties than it resolved, it has provided a benchmark for further large clinical trials that can answer questions about the effectiveness of different fluid regimens, including albumin, as volume expanders.

In addition to issues of relative effectiveness between these fluid solutions, cost is also an important part of the debate, because colloids are much more expensive than crystalloids. Higher costs increasingly should be justified in terms of greater benefit to patients. Because large volumes of intravenous fluids are used, even small price differences are economically important. Not only are human albumin solutions the most expensive colloid solutions, but also they are produced from blood (with all the associated risks). Their use requires the scrutiny of properly conducted clinical research.

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## Disclaimer

The authors have disclosed no conflicts of interest.

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## 21

# Applications of Cellular Radiolabeling in Transfusion Medicine

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The ability to track the fate of injected cells can provide very useful information in the care of patients, in clinical trials of new blood component production or storage, and in investigation of new cellular therapies. The ability to distinguish the injected cells from the vastly larger quantity of autologous cells that have been circulating allows the determination of their short-term recovery and long-term survival, identification of their destination or site of destruction, or provision of clues regarding their distribution and function. The techniques used for radiolabeling are generally well established, and the methods for determining the kinetics of the cells have been standardized. This chapter provides an overview of these techniques and methods with the intentions of promoting standardization of practices and investigation of alternative approaches as well as broadening the application of these techniques.

The techniques described are those that have used labeling, either *ex vivo* or *in vivo*, to determine the fate of the cells of interest. Alternative, nonradioactive labels have also been developed, but are currently used only in research settings. Their application is also mentioned briefly. Although all of these techniques need to be applied carefully and according to protocol in order to generate reliable outcomes, their application is well within the grasp of most laboratories having the appropriate equipment and competent staff to conduct careful laboratory determinations. Their utility suggests that a broader application could provide additional, useful information to both clinicians and researchers.

## Concepts of Recovery Curves and Kinetic Analyses

There are multiple specifications for a useful and practical radiolabeling agent that is to be applied in human clinical trials.

No single radioisotope possesses optimal characteristics in all categories, so trade-offs are inevitable. The requirements listed below appear in no specific order. All are important and, yet, the failure to achieve the greatest benefit from use of any one element does not preclude a radiolabel's use. Specific problems or limitations may be circumvented through a variety of techniques.

## Recipient Concerns

Whether the recipient is a healthy volunteer receiving an aliquot of autologous cells or a patient undergoing a diagnostic study, minimization of the toxicity or potential harm to the individual is the prime requirement. Safety must be viewed in terms of the injected dose of the radioisotope as well as the persistence (half-life) of the isotope and the disposition of the radionuclide and any accompanying chelating agent. A "low" dose of a radionuclide with a long half-life may yield more exposure than a higher dose of an isotope with a short half-life. The site of sequestration of labeled cells or of storage of the radionuclide may serve to concentrate the radiation exposure in certain tissues. More latitude for potential toxicity might be regarded as acceptable if the recipient is a patient who will receive benefit through the diagnosis rather than if the recipient is a healthy volunteer participating in a clinical trial. Nevertheless, the principle of limiting exposure (as low as reasonably achievable, or ALARA) pertains to the radiation exposure received by any recipient.

## Radioisotopic Properties

Key properties of radioisotopes include half-life, distribution, and energy of emissions. The rate of decay should allow for tracking the cells over a sufficient time to answer the question at hand without exposing the recipient to unnecessarily protracted radiation. For example, determining the red cell volume (or mass) can readily be accomplished with <sup>99m</sup>Tc (with a 6-hour half-life) because the calculations depend only on the initial dilution. A longer-lived radioisotope, such as <sup>51</sup>Cr (with a 27.7-day half-life) is necessary for determining recovery at 24 hours and for determining survival over multiple weeks.

The distribution of the isotope is important not only for exposure and toxicity concerns as mentioned above but also for ensuring that the radioactivity being measured in the blood is associated with the expected cells. At what rate does the radiolabel elute from the labeled cells? On elution or release with lysis, can the label be taken up by other cells? Once released from a cell, does the label persist in plasma? (These last two issues affect the extent to which radioactivity found in a blood sample can be attributed to the labeled cells that were administered.) The energies of the emissions that occur on decay are also important. A gamma counter must be set to detect photons in the energy ranges (“windows”) associated with a particular isotope. The device must also be able to distinguish the presence of two different isotopes for simultaneous injection of two different populations of cells. (For example, the major gamma-photon emissions of  $^{51}\text{Cr}$  and  $^{99\text{m}}\text{Tc}$  permit simultaneous counting of these radionuclides.) Radionuclides with high emission energies may, paradoxically, have a low counting efficiency unless the gamma counter has a large NaI crystal that can intercept such photons and translate their energy to visible light detectable by the photomultiplier system. Radioisotopes with higher energy emissions are more useful in external imaging when location of the injected cells is important, such as when radiolabeled granulocytes are used to identify the site of an abscess.<sup>1-3</sup>

### Cellular Function

The metabolic impact of the radionuclide itself and any carrier molecule to which it may be bound or chelated is important to know in order ensure that the labeling process is not altering the cell's physiology. Besides significant direct toxicity that kills the cell and thus prevents its circulation and normal function, subtle derangements of function may have significant impacts on disposition of cells, such as alteration of the chemotactic response affecting the ability of a neutrophil to track to a site of infection.<sup>4</sup>

### Specificity

The more specific the radiolabel is for a particular cell type, the less prelabeling purification will be required. Similarly, the better the cells' uptake of the label, the less postlabeling washing will be necessary to ensure that the label being injected is contained in the (intended) cells. At the same time, consideration must be given to the relative uptake of label by different subpopulations. Are cells of all ages able to take up and retain the label equally? Does preferential labeling of a certain subpopulation occur?

### Procedural Issues

The issues above combine to determine the extent to which the sample will need to be manipulated. In general, better retention of cellular function can be predicted with fewer manipulations. This is more of an issue for platelets or granulocytes than for red cells. However, even seemingly innocuous steps can induce cell rupture and hemolysis or other effects that may not be experienced uniformly across the entire population of cells and thus yield a skewed subpopulation for labeling and/or infusion.

Furthermore, although procedures can be performed in laminar flow hoods to reduce the potential for bacterial contamination, using closed systems wherever possible and reducing the number of steps employed are to be encouraged.

### The Radiolabeling Process

The steps of radiolabeling procedures are similar for red cells, platelets, and granulocytes—purification, preparation, uptake, wash. Purification improves accuracy by limiting the radiolabeling of cells that might confuse the observation of the cells of interest. The necessary degree of purification is inversely proportional to the expected life span of the infused cells and directly proportional to the relative number of the target cell type among other cell types. For example, the number of platelets or leukocytes in a red cell preparation would be intrinsically low (even without leukocyte reduction filtration or differential centrifugation) because of their relative proportion in circulation, and their shorter circulatory half-lives would not be expected to perturb the red cell recovery curve significantly. However, red cell content in a suspension of platelets or granulocytes might dramatically alter the observed recovery and survival of the platelets.<sup>5</sup>

The proclivity of the radionuclide to label the contaminating cells in comparison with the target cell type also needs to be considered. Certain labels, such as  $^{111}\text{In}$ , require a plasma-free medium to avoid binding of the cation to transferrin, a problem that has a beneficial side in that a plasma wash at the end of the labeling quickly removes all of the radiolabel that is not intracellular.

Once the cell has taken up the label, various interactions occur to reduce the rate at which it diffuses back out (“elution”).  $^{51}\text{Cr}$  binds to the  $\beta$ -globin chain of hemoglobin, but  $^{99\text{m}}\text{Tc}$  first requires reduction from pertechnetate ( $\text{TcO}_4^-$ ) to  $\text{Tc}^{+4}$ . To do this, red cells are pretreated with stannous chloride (commonly referred to as tinning) to create a reducing environment that allows for this “capture” of the radiolabel. Tin pyrophosphate can also be administered to patients directly followed by  $^{99\text{m}}\text{Tc}$  to effect red cell labeling in vivo for scanning studies, but the efficiency achieved is lower than the 90% to 95% seen with ex-vivo labeling.<sup>6</sup>

$^{111}\text{In}$ , used most frequently in platelet and granulocyte labeling, does not bind to an intracellular target but is trapped in the cell on diffusion of its lipophilic carrier. The  $^{111}\text{In}$  chelate (such as indium oxine or indium tropolone) dissociates in a dynamic fashion and, when this occurs, the carrier can diffuse out of the cell again, thus trapping the  $^{111}\text{In}^{+3}$  inside. Following removal of radiolabel that has not been taken up by cells, the cells should be infused as soon as possible, and, for improved accuracy of the recovery calculation, the proportion of radiolabel within the cell as opposed to the supernatant should be determined concurrently with the infusion.<sup>7</sup>

### Determination of Recovery

The recovery of the injected cells can be calculated in one of several ways.<sup>8</sup> The simplest approach uses a calculated blood volume

(based on the subject's height, weight, and gender,<sup>9,10</sup> for example) and then applies the total amount of cellular radioactivity injected to determine the 100% recovery point. This approach is commonly used in platelet studies where a large proportion (usually at least 30%) of the label is retained in the spleen and the extrapolation method (described next) would not be accurate. It is also helpful in platelet labeling because separation of platelets in a blood sample in order to express their relative volume (a thrombocrit) is not usually feasible, and thus the relevant calculations cannot yield a "total platelet volume" (or mass) for the subject. The accuracy of this approach, however, is limited to the accuracy of the prediction of the subject's blood volume. Most formulae or nomograms display substantial inaccuracy, especially as the weight of the subject diverges from average.

A second approach depends on a back-extrapolation from the first several samples taken after infusion to the time of injection ( $T_0$ ) to identify what the activity of the blood would have been had the labeled cells instantly been uniformly mixed throughout the subject's blood volume. This establishes the 100% recovery point to which can be compared the actual recovery at later times. This is commonly referred to as the single-label recovery, or SLR. With knowledge of the total cellular amount of radioactivity injected, that activity can also be used to calculate the volume of distribution of the label.

Inherent in this calculation is the assumption that the disappearance of the labeled cells over the time used to create the curve from which the extrapolation to  $T_0$  is based, is the same as the kinetics of the cells before the first sampling. Even when sampling begins quite promptly after infusion (eg, 5 minutes), very rapid removal of a substantial proportion of labeled cells (such as through their accumulation of a lethal storage lesion, for example) would cause the extrapolation to miss this event and place the  $T_0$  point at too low a level.<sup>11</sup> This then overestimates the volume of distribution and may lead to an erroneously high estimation of the recovery. This approach is generally accepted as accurate when the immediate recovery exceeds 70% and possibly at even lower levels.

An alternative approach that would be expected to provide the best accuracy uses a second, independent method to establish the blood volume and then applies that determined volume to calculate the  $T_0$  on the basis of the observed dilution. (This is commonly referred to the double-label recovery, or DLR, method.) A freshly collected sample of red cells from the subject is labeled with a different radionuclide than that used for labeling the cells that are the subject of the study, that is, the "test" cells. Some have used albumin labeled with radioactive iodine for determination of blood volume, but the loss of the label and the distribution of the albumin molecules have raised questions about the dependability or advisability of this technique.<sup>8,12</sup> Because the volume-setting cells are fresh and autologous, the assumption is made that they will all circulate after reinfusion, and thus extrapolation of the recovery curve to  $T_0$  (using an SLR-like method) or an average of the first several recovery points as "100% recovery" will accurately determine the blood volume. In turn, this volume,

when divided into the total cellular radioactivity reinfused in the test cells, sets the 100% recovery point for the test cells. Any early loss of the test cells will not affect the recovery calculation because the  $T_0$  activity is not dependent upon extrapolation of the test cells' recovery curve. This approach reduces the error in blood volume determination to around 2%, less than half that seen with calculations based on height and weight.<sup>8</sup>

The DLR approach has several drawbacks, however. Most important is the extra radiation exposure of the subject. Although avoidance of exposure to unnecessary radiation is always desirable, the actual additional exposure is small in most red cell recovery studies. The usual isotope used for the freshly collected autologous cells (approximately 10  $\mu$ Ci  $^{99m}\text{Tc}$ ) adds <25% to splenic exposure and <4% to total body exposure compared to that imparted by longer-lived  $^{51}\text{Cr}$  used to label the test cells.<sup>13</sup> In addition, although the subject must be available for an additional phlebotomy to start the labeling process, a sample is usually collected the morning of reinfusion anyway in order to confirm the absence of a pregnancy via a serum test in females of childbearing potential. There is no doubt, however, that the DLR approach is more expensive, as it takes more time to complete the labeling and uses more supplies and a second radionuclide.

Is application of the DLR approach justified or necessary? The difference in single-label vs double-label red cell recoveries for most collection and storage systems that are evaluated in clinical trials is relatively small, on the order of 1% to 2%.<sup>14,15</sup> The direction of the difference that has been observed, however, is not what might have been predicted; that is, DLR is usually slightly higher (when looking at the mean for a clinical trial) than SLR. The reason for this difference remains to be elucidated but may be related to the early elution of  $^{99m}\text{Tc}$  from the freshly collected cells and clearance from circulation or less-than-complete recovery of the fresh cells. Perhaps even more important than the accuracy, however, is the objective determination of blood volume and thus  $T_0$  activity that DLR provides. The fitting of the SLR line to the initial points of the test cell's recovery curve is always subject to interpretation, and decisions must be made as to which points appear to be outliers and thus should be excluded. The DLR approach provides an objective determination of the 100% recovery point, at the least verifying the SLR  $T_0$  extrapolation or, in cases where this projection appears to be erroneous, providing an alternative or "back up" method to complete the calculations. Currently, the Food and Drug Administration (FDA) does not specify the calculation method (SLR vs DLR) to be used in clinical trials of red cell storage systems, but most laboratories prefer to use DLR for the reasons cited above.

The DLR approach of determining blood volume based on dilution of a known activity in the injectate is the method used to determine red cell volume in patients suspected of having polycythemia. Using  $^{99m}\text{Tc}$  to label autologous cells (to limit radiation exposure) and reinfusing them promptly, an average of the first points on the recovery curve (ie, 5, 7.5, and 10 minutes) are usually applied to determine the patient's red cell volume.

Also, as in DLR, the assumptions of 100% recovery of red cells and lack of any elution of radiolabel are made. This is compared to a reference range based on gender, height, and weight in order to determine whether the red cell volume is indeed elevated or whether a pseudopolycythemia exists (Gaisbock syndrome) in which the hematocrit is elevated because of a reduction in plasma volume.

For all of these calculations, additional accuracy is achieved by determining the proportion of the injectate's radioactivity that is cell-bound at the time of infusion.<sup>7</sup> This is accomplished by centrifuging an aliquot of the injectate at the same time as the reinfusion and multiplying the cellular fraction of activity (usually 95-99%) by the total radioactivity of the injectate.

The in-vivo survival of red cells beyond 24 hours is not routinely included in precensure clinical trials. Instead, it is assumed that cells able to survive for the first 24 hours after reinfusion will have a normal life span, as has been seen in most of the studies where this information has been sought. However, survival may be an important parameter to consider when evaluating an intervention that has the potential to modify red cell biochemistry.

In calculating the survival,<sup>16</sup> the recovery at each time point,  $N_t$ , is expressed in counts/minute/g of hemoglobin after correction for background, decay, and elution of the radiolabel from the red cell. The elution rate for  $^{51}\text{Cr}$  is approximately 1%/day but is slightly higher over the first 10 days, thus leading to recommendations for application of the elution correction factors developed by Mollison.<sup>17-20</sup> The fractional recovery,  $N_t/N_0$ , is then calculated at each time point where  $N_t$  = counts at time "t" corrected for elution and  $N_0$  = counts at time "zero." The survival curve may not be linear, and three possible regression models have been recommended,<sup>16</sup> including:

$$N_t/N_0 = \alpha * e^{-\beta * t} + (1 - \alpha) e^{-\gamma * t} \text{ (exponential; two component)}$$

$$N_t/N_0 = \alpha * e^{-\beta * t} + \gamma \text{ (exponential)}$$

$$N_t/N_0 = \alpha + \beta * t \text{ (linear)}$$

where  $\alpha$ ,  $\beta$ , and  $\gamma$  are arbitrary fitting parameters. Of these, the linear approach is generally used. The exponential models are useful for nonlinear survival curves when a random clearance mechanism is operating in addition to senescence. The mean life span is the projected x-axis (time) intercept of the tangent to the curve at  $t = 0$ . The  $T_{50}$ , the time required for half of the red cells to disappear, is reported.

The  $T_{50}$  does not bear a simple relationship to the mean life span.<sup>17</sup> Modest reductions in the  $T_{50}$  can correlate with a substantial increase in the rate of red cell destruction.<sup>21</sup> The  $T_{50}$  is usually best approximated with a log-linear extrapolation after correcting for elution of the label from the cells.

For platelets, the calculations of recovery and survival are undertaken in a slightly different manner. The currently accepted approach specifies that the recovery at time points beyond 20 hours are used for nonlinear curve fitting to estimate recovery

and survival.<sup>22</sup> The multiple hit model is most commonly used because of its congruence with the platelet kinetics found in normal subjects.<sup>23</sup> The recovery is determined by extrapolation back to  $T_0$ , analogous to red cell SLR studies. The "survival" parameter is usually taken as the numerical expected life span, the point at which a tangent to the survival curve crosses the x-axis. This value is very sensitive to the first portion of the curve (which usually includes more variance than the later recovery points), and thus the values obtained in the first 20 hours are not included in the curve fitting. These parameters may be estimated using a nonlinear curve fitting routine found in various statistical and pharmacokinetic computer program packages. (Most laboratories use the COST software,<sup>24</sup> but the calculations provided by commercially available software packages can produce identical results.<sup>25</sup>)

## Radionuclides Used in Kinetic Determinations

The first clinically useful red cell radiolabel,  $^{51}\text{Cr}$ , was described by Gray and Sterling<sup>26</sup> in 1950. This isotope was quickly identified as an excellent red cell label for survival and recovery studies.  $^{99m}\text{Tc}$ <sup>27</sup> and  $^{111}\text{In}$  were subsequently recognized as useful radiolabels of red cells, and McAfee and Thakur<sup>28</sup> in 1976 demonstrated  $^{111}\text{In}$  to be an effective radiolabel of leukocytes. Other cell radiolabels have also been evaluated and used, with varying degrees of success. However, the vast majority of blood cell kinetic and imaging studies are performed with  $^{51}\text{Cr}$ ,  $^{99m}\text{Tc}$ , or  $^{111}\text{In}$ .

Investigators should have an understanding of fundamentals of nuclear medicine and radiation biophysics to use these materials appropriately. Desirable characteristics relate to recipient safety as well as to ease and reproducibility of use (Table 21-1). Each of the three commonly used radionuclides has advantages and disadvantages that affect their applicability in clinical and research settings.

The selection of an appropriate radionuclide requires familiarity with the radioactive half-life, elution characteristics, and gamma-photon energy of  $^{51}\text{Cr}$ ,  $^{99m}\text{Tc}$ , and  $^{111}\text{In}$ . These features are listed in Table 21-2. The optimal range for detection of photoelectric events in a standard gamma counting instrument (gamma counter) is 100 to 300 keV. Thus, gamma-photon

**Table 21-1.** Desirable Features of Blood Cell Radiolabels

- Minimal manipulation of cells required during preparation
- Specific for the cell being studied
- Nontoxic to the labeled cell
- Nontoxic to the recipient
- Minimal radiation dose to the recipient
- No metabolism of the label by the cell
- No elution of the label
- Radioactive half-life appropriate for the duration of the study
- Radioactive emissions suitable for efficient detection or imaging
- No relabeling of other cells in vivo

**Table 21-2.** Major Features of Radionuclides Used for Blood Cell Labeling

|                                  | <sup>51</sup> Cr | <sup>99m</sup> Tc | <sup>111</sup> In          |
|----------------------------------|------------------|-------------------|----------------------------|
| Radioactivity half-life          | 27.7 days        | 6.0 hours         | 2.8 days                   |
| Major γ-photon emissions (yield) | 320 keV (9.8%)   | 140 keV (90%)     | 173 and 247 keV (90%, 94%) |
| Elution rate (red cells)         | 1% per day       | 1%-7% per hour    | 4%-8% per day              |
| Suitable for imaging?            | No               | Yes               | Yes                        |

energies in this range are desirable. The “yield” of gamma-photon emissions is the number of photons emitted per 100 radioactive decays. A high yield means that a lower radiation dose can be used to achieve adequate detection levels.

**Chromium-51**

<sup>51</sup>Cr can be used to label both red cells and platelets. Advantages of this radionuclide as a red cell label include ease of labeling, excellent uptake, low toxicity, and a low and stable elution rate. It is the standard against which other red cell labels are compared. <sup>51</sup>Cr is produced in a reactor by neutron activation. It decays by electron capture, with a radioactive half-life of 27.7 days. The principal gamma-photon emission occurs by electron capture at 320 keV, slightly above the optimal detection range of standard gamma counters. The high energy and low yield (9.8%) of gamma-photon emissions from decay of this radionuclide necessitate a larger NaI crystal (typically 3-inch) for efficient detection in a gamma counter. Smaller crystals require a larger dose of the radionuclide to achieve adequate counts.

The 27.7-day half-life of <sup>51</sup>Cr makes it suitable for most red cell survival and recovery studies. However, if multiple studies are performed, the overlapping survival curves make separation of the individual studies quite difficult. This problem can be addressed in recovery studies by correcting for residual radiation, by separating studies until the nuclide decays, or by using alternate radionuclides.

<sup>51</sup>Cr is supplied as sodium radiochromate (Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub>) for cell labeling purposes. Hexavalent chromate labels red cells by binding rapidly and reversibly to the red cell membrane. After reduction to the trivalent state, the chromic ion binds more slowly and firmly to the β-globin chain of intracellular hemoglobin, and probably to other intracellular ligands as well. Younger red cells take up slightly more label than do older cells. The bond is quite firm, and any unbound trivalent (reduced) chromium does not label other red cells. Labeling efficiency is about 90%.

Chromium elutes from red cells in two phases. There is an early loss of 1% to 4% of the label within 24 hours, which may represent elution of a loosely bound fraction of the label.<sup>29</sup> Garby and Mollison<sup>17</sup> found that subsequent elution varies from 0.56% to 2.04% per day, similar to the 0.70% to 1.55% reported by Bentley and associates.<sup>30</sup> An estimated elution rate of 1% per day is satisfactory for most investigational and clinical purposes. Trivalent chromium that elutes from the labeled cells is rapidly excreted in the urine and does not relabel other cells in vivo.

The technical steps for the labeling of red cells with <sup>51</sup>Cr for the evaluation of blood storage and preservation systems have been described by Moroff and colleagues.<sup>31</sup> The International Committee for Standardization in Hematology<sup>16</sup> has described a slightly different technique suitable for labeling red cells for the determination of transfusion compatibility. A nonradioactive isotope of chromium has also been investigated as a red cell label. <sup>52</sup>Cr-labeled red cells have in-vivo survivals similar to red cells labeled with <sup>51</sup>Cr,<sup>32</sup> but the isotope must be detected by atomic absorption spectroscopy, making it impractical for clinical use.

Baldini and colleagues<sup>33</sup> first described the use of <sup>51</sup>Cr as a platelet radiolabel. The platelet-labeling efficiency of <sup>51</sup>Cr is low (9%) compared with its red cell labeling efficiency (90%). Therefore, it is important that platelet preparations be free of red cells before being labeled. A procedure for labeling platelets with <sup>51</sup>Cr, which is more difficult than that for red cells, has been described by Snyder and colleagues.<sup>34</sup> Further useful refinements were developed by Heaton, Holme, and colleagues.<sup>8,35</sup> <sup>51</sup>Cr is not a useful isotope for imaging studies, which require a high yield of gamma-photon emissions.

**Technetium-99 m**

<sup>99m</sup>Tc, which is widely employed as an imaging agent in nuclear medicine, has characteristics that are different from <sup>51</sup>Cr, making it a useful adjunctive red cell radiolabel. This metastable radionuclide is produced in a molybdenum generator as a product of molybdenum-99 decay. <sup>99m</sup>Tc decays with a half-life of 6.02 hours to <sup>99</sup>Tc. <sup>99m</sup>Tc emits a gamma-photon at 140 keV, which falls within the optimal range of gamma counters. The high yield (90%) of this radionuclide is useful for external imaging. This radionuclide is widely used as an imaging agent because of the ease of preparation, high yield, and easy detectability. Because of the frequent use of this radionuclide, many hospitals maintain a generator on site, and thus there is a readily available and inexpensive source of <sup>99m</sup>Tc for cell labeling applications.

<sup>99m</sup>Tc, as pertechnetate, diffuses across the red cell membrane into the cell, where it is reduced and binds to hemoglobin and other intracellular ligands with a labeling efficiency of about 90%. The short half-life of <sup>99m</sup>Tc makes it suitable for short-term studies of red cell mass but precludes its use for long-term red cell survival studies. Groups of samples must be counted rapidly, and a correction must be made for radioactive decay that occurs during the counting procedure. However, because of its short

half-life,  $^{99m}\text{Tc}$  may be useful as a red cell label when a series of survival studies must be performed for transfusion compatibility. It is also useful for the accurate determination of red cell volume as part of red cell recovery studies (using  $^{51}\text{Cr}$ ) or in patients being investigated for possible pathologic expansion of their red cell mass (eg, polycythemia).

This nuclide also has a high and variable rate of elution, which reduces precision in all but short-term studies.<sup>36,37</sup> The use of stannous compounds as intracellular reducing agents in the labeling process has permitted firmer binding of  $^{99m}\text{Tc}$ , but elution rates of 4% per hour with considerable variation may be expected. Heaton and colleagues<sup>7</sup> have described the technical steps necessary to minimize variability in labeling and elution with this nuclide.

### Indium-111

$^{111}\text{In}$  is a useful label for red cells, platelets, and leukocytes. The radioactive half-life of the nuclide is 2.83 days, making it appropriate for red cell mass determinations, platelet recovery studies, and leukocyte imaging.  $^{111}\text{In}$  is prepared in a cyclotron by proton bombardment of a cadmium target. It has two major gamma-photon emissions, 173 keV (90.5% yield) and 247 keV (94% yield), both of which are excellent for counting in a gamma counter and for external imaging.

$^{111}\text{In}$  must be complexed with a lipophilic chelating agent to enable it to cross cell membranes and label intracellular proteins. Although several lipophilic chelates have been studied, 8-hydroxyquinoline (oxine) is the only agent currently licensed for this purpose in the United States. Technical procedures have been published for labeling red cells,<sup>38</sup> platelets,<sup>34</sup> and leukocytes<sup>28</sup> with  $^{111}\text{In}$ . It is a nonselective label, preferentially taken up by platelets, with less by taken up by leukocytes, and still less by red cells.<sup>39</sup> Therefore, cell preparations must be free of extraneous elements before being labeled.  $^{111}\text{In}$  has a high and variable rate of elution from red cells.<sup>38</sup> Approximately 8% to 10% of the label elutes within 24 to 48 hours, with a subsequent loss of 4% per day. This renders  $^{111}\text{In}$  unsuitable for red cell survival and recovery studies, but it is useful for determining red cell volume when precision is not critical.

## Radiolabeling as a Tool for Evaluation of Cellular Kinetics and Traffic

### Red Cells

Short-term red cell recovery studies are useful for the identification of patterns of immune red cell destruction and for evaluating new methods of storing and preserving the cells. The normal red cell life span (110 to 120 days) was first approximated by differential absorption techniques<sup>40</sup> and subsequently confirmed by radioisotopic labeling studies using cohort (marrow or in-vivo) labels<sup>41</sup> and random (peripheral blood) labels.<sup>17</sup> When  $^{51}\text{Cr}$  is used as the label, the time at which the recovered counts are 50% of the originally injected counts is the  $T_{50}\text{Cr}$ , normally

$31 \pm 6$  days. Correction for elution will yield the true red cell life span. Modest reductions in the  $T_{50}\text{Cr}$  can indicate a substantial increase in the rate of red cell destruction.<sup>21</sup> The  $T_{50}\text{Cr}$  is also useful for evaluating the extent of hemolysis in a chronic hemolytic anemia.

Red cell life span studies also have been conducted to assess the therapeutic benefit of transfused units that are enriched in young red cells ("neocytes"). The transfusion of such units would have the theoretical benefit of reducing the iron burden in patients who undergo frequent transfusions by increasing the number of longer-lived red cells in the circulation.<sup>42</sup> Neocytes survive 30% to 60% longer in the circulation than do red cells derived from standard Red Blood Cell (RBC) units.<sup>43,44</sup> However, as neocyte units contain only about half the hemoglobin of standard units and are costly to prepare, they have not achieved wide clinical acceptance.<sup>45</sup>

In contrast to long-term life span studies, short-term red cell survival studies are useful in identifying patterns of immune red cell destruction. A careful assessment of the mechanism of red cell destruction may be necessary when an unexplained transfusion reaction has occurred or when standard pretransfusion serologic testing has not clearly identified or characterized red cell allo- or autoantibodies in a transfusion recipient. Furthermore, hemolytic transfusion reactions are not always immunologically mediated. In addition, patients with serologic findings suggesting a hemolytic reaction may not demonstrate clear evidence of clinical hemolysis.<sup>46,47</sup> In-vivo studies may be useful to identify accelerated red cell destruction when present and to identify characteristic recovery patterns that can influence subsequent transfusion decisions.

The factors that contribute to immune red cell destruction have been discussed in detail elsewhere.<sup>48,49</sup> Of primary importance are the immunoglobulin class and subclass of the recipient red cell alloantibody<sup>50</sup> and the extent to which that antibody activates complement.<sup>51,52</sup> The important role of cytokines in IgG-mediated red cell destruction has also been clarified.<sup>53,54</sup> Short-term (24-hour) recovery studies using fresh allogeneic red cells are performed for transfusion compatibility. A red cell phenotype is chosen on the basis of serologic results. The cells are usually labeled with  $^{51}\text{Cr}$ . If serial studies are required, a radionuclide with a shorter half-life,  $^{99m}\text{Tc}$  or  $^{111}\text{In}$ , can be selected, but problems with rapid elution of the label and overlapping counts may complicate interpretation of the results.

Red cell recovery curves observed in 24-hour survival studies conducted to determine transfusion compatibility fall into four characteristic patterns (Fig 21-1). They are 1) normal survival, 2) extravascular destruction characterized by a "two-component" survival curve, 3) extravascular destruction characterized by a "single exponential" survival curve, and 4) intravascular destruction.

### Normal Recovery

In a normal subject the recovery of fresh, compatible red cells is 97% to 102% at 60 minutes and 95% to 100% at 24 hours.



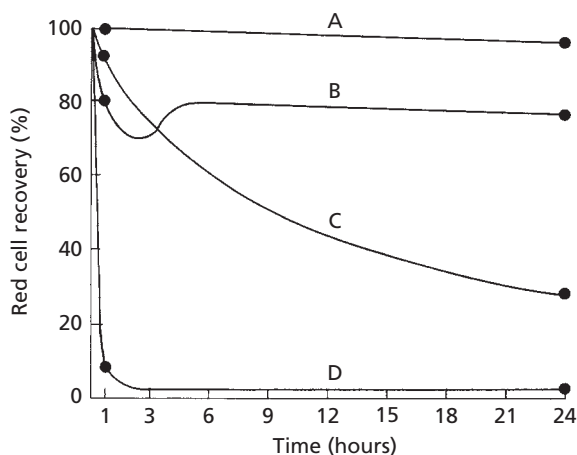


**Figure 21-1.** Red cell recovery curves 24 hours after transfusion show differing mechanisms of immune red cell destruction. (A) Normal recovery. (B) Partial complement activation with two-component extravascular destruction pattern (eg, IgM anti-Le). (C) No complement activation with single exponential extravascular destruction (eg, IgG anti-D). (D) Complete complement activation with intravascular destruction (eg, anti-A). The 1-hour recovery percentage is not always predictive of transfusion safety.

### Extravascular Destruction without Complement Activation (Single Exponential Survival Curve)

Most IgG antibodies do not activate complement. Instead, there is progressive removal of sensitized red cells with a survival curve described by a single exponential. The concentration of the antibody, its IgG subclass, and the generation of inflammatory cytokines are important determinants of the rate of removal of the cells. The four IgG subclasses vary in their efficiency to bind to Fc receptors on reticuloendothelial system (RES) macrophages.<sup>55</sup> IgG3 is often associated with immune hemolysis, while IgG2 and IgG4 are inefficient in removing sensitized cells. IgG1, the most common subclass, varies in its ability to remove red cells. IgG1 is present in higher titer than the other subclasses and is most often involved in IgG-mediated red cell destruction.

Antibodies of the Rh, Kell, Duffy, and Kidd blood systems are usually IgG and do not activate complement. The 60-minute red cell recovery in the presence of these antibodies may exceed the 70% minimum required for transfusion safety, but may fall well below this standard at 24 hours. This pattern of red cell destruction highlights the importance of extending red cell recovery studies to at least 24 hours. Transfusion based on the 60-minute recovery percentage alone may place the patient at risk for accelerated red cell removal.

### Extravascular Destruction with Partial Complement Activation (Two-Component Recovery Curve)

The transfusion of incompatible red cells may activate complement. Most IgM red cell alloantibodies and some IgG alloantibodies (eg, anti-Jk<sup>a</sup>) can initiate the complement cascade. IgM antibodies directly activate complement, with 20 to 40

IgM molecules per red cell being required to initiate red cell clearance. IgG antibodies can activate complement if two or more molecules are physically adjacent on the red cell membrane.<sup>56</sup> Thus, many more IgG molecules are required for complement activation to occur. In most cases, the kinetics of complement activation result in generation of the membrane-bound C3b fragment but not the final C5b-C9 “membrane attack” complex. C3b is subject to cleavage to C3bi by plasma factor I, with plasma factor H as a cofactor. Red-cell-bound C3b and C3bi adhere to the complement receptors CR1 and CR3 on RES macrophages in the liver and spleen.<sup>57</sup> These adherent red cells may be removed from the circulation by either antibody-dependent cell-mediated cytotoxicity or complete phagocytosis. They may also sustain membrane damage and spherocytic change after partial phagocytosis.

Only a minority of adherent red cells are damaged or destroyed by these mechanisms. C3bi may be degraded before red cell damage occurs. Plasma factors I and H further cleave C3bi to C3c, which detaches from the cell, and C3d,g, which remains attached to the cell membrane.<sup>58</sup> C3d,g-coated red cells do not effectively bind to complement receptors of the RES. Most red cells coated with C3d,g detach from RES macrophages and survive relatively normally.<sup>59</sup> The inactive C3d,g fragments may “protect” the red cell from further complement-mediated damage by occupying complement-binding sites. A “two-component” recovery curve, therefore, demonstrated the early loss of C3b-coated red cells and more normal survival of those cells on whose membranes C3b has degraded to C3d,g. Red cell antibodies that usually react optimally at temperatures below 37°C (eg, Lewis, MNS, P) will often yield “two-component” recovery curves. A “two-component” survival curve with more than 70% recovery at 24 hours indicates that the patient can safely receive larger quantities of similar red cells with little risk of acute immune hemolysis.

### Intravascular Destruction

IgM antibodies that are efficient activators of complement, such as anti-A and anti-B, can drive the kinetics of the reaction to the formation of the terminal C5b-C9 “membrane attack” complex. Insertion of this complex into the red cell membrane results in loss of membrane integrity and osmotic lysis of the cell. Transfused cells that generate complement activation of this magnitude are usually hemolyzed within minutes.

### Synergistic Effect of Complement and IgG

Selected antibodies of the IgG class, such as Kidd antibodies, activate complement. Complement and IgG appear to act synergistically in promoting red cell opsonization and destruction. The addition of complement to red cells sensitized with IgG alone will enhance phagocytosis.<sup>60,61</sup> A hybrid red cell survival curve may occur when both IgM and IgG antibodies are active. There is a rapid loss of a minor population of the cells from IgM-mediated complement activation, and a slower phase of cell destruction mediated primarily by the IgG antibody.

## Platelets

Studies of platelet life span and kinetics were originally performed with  $^{51}\text{Cr}$ .<sup>62</sup> However, the low labeling efficiency, long half-life, and poor imaging characteristics of  $^{51}\text{Cr}$  have led to preferential use of  $^{111}\text{In}$  when a single platelet label is required.<sup>63</sup> Serial studies in a subject using a single label may not be possible, however, as platelet kinetics vary over time. A reduction in this variability is seen with use of both chromium and indium labels for simultaneous infusion. Thus, a more accurate estimation of the difference between platelets stored in a standard (or licensed) system and those in a test system can be achieved.<sup>64</sup> This technique has also been used to create novel approaches to platelet storage studies. In one such experiment, comparing whole-blood-derived platelets stored for 5 days with those stored for 7 days, the test (7-day) platelets were derived from a unit collected on Day 0, and the control (5-day) platelets were derived from a collection 2 days later. Platelets from both study arms were labeled and infused simultaneously using the approach of two different radiolabels.<sup>65</sup>

Standard technical methods for preparation of radiolabeled platelets have been published.<sup>22,35</sup>  $^{111}\text{In}$ -oxine has a high affinity for red cells, leukocytes, and plasma proteins, primarily transferrin. Thus, washing and centrifugation steps are necessary before labeling to prepare platelets that are free from plasma and other cellular components. These steps, however, cause a substantial loss of platelets (~40%) and a “collection injury” that can distort test results if not performed carefully. Other compounds that form lipophilic complexes with  $^{111}\text{In}$ , notably acetylacetone,<sup>66</sup> tropolone,<sup>67</sup> and 2-mercaptopyridine-N-oxide (merc),<sup>68</sup> have been studied as possible alternative agents to overcome this problem. Tropolone is widely used in Europe, but oxine remains the only licensed agent in the United States.

$^{111}\text{In}$  begins eluting from platelets shortly after labeling and infusion. Heaton and colleagues developed a technique for determining the elution characteristics.<sup>7</sup> Application of this “elution correction” has shown that the recovery curves for platelets labeled with  $^{111}\text{In}$  are coincident with those labeled with  $^{51}\text{Cr}$ . This procedure is the basis of the standard platelet radiolabeling protocol that has been adopted by the Biomedical Excellence for Safer Transfusion (BEST) Collaborative. Murphy proposed fresh platelets as controls for assessing platelet recovery, thus avoiding “creeping inferiority” that may occur if platelets stored with standard methods are used as controls in sequential studies.<sup>69</sup>

The calculation of platelet life span can be performed by four different methods—linear, exponential, weighted mean, and  $\gamma$ -function (multiple hit)—each of which yields slightly different results. The  $\gamma$ -function analysis is the recommended method,<sup>34</sup> and is the most frequently used. Investigators often report their results using one or more additional statistical methods. Computer programs have been developed that model platelet survival by a variety of techniques,<sup>24,70</sup> or statistical analysis software can be used.

No radioactive tracer selectively labels newly formed platelets. Thus, cohort studies have not been practical to measure platelet

production. Instead, the kinetics of normal platelet production have been measured indirectly by determining the turnover rate of circulating platelets (platelet count divided by platelet survival corrected for recovery). Estimates of daily platelet production in the steady state using this method have ranged from 35,000 to 66,000/ $\mu\text{L}$  of blood.<sup>71</sup> The life span of human platelets ranges between 8 and 12 days; Harker and Finch<sup>72</sup> found it to be  $9.5 \pm 0.6$  days. Senescent platelets are removed from the circulation by RES macrophages in the liver and spleen and, to a lesser extent, by marrow and lungs.<sup>73,74</sup>

In addition to platelets removed through senescence, there is a fixed platelet requirement necessary to maintain vascular integrity. Hanson and Slichter<sup>75</sup> have demonstrated that approximately 82% of platelet turnover in normal persons results from senescence, and 18% (~7100 platelets/ $\mu\text{L}/\text{day}$ ) is due to the fixed requirement. This fixed requirement becomes an increasingly large component of platelet removal as thrombocytopenia from marrow hypoplasia worsens. Thus, an accelerated reduction in life span is noted as platelet counts decrease below 50,000/ $\mu\text{L}$ . The shortened platelet life span observed in thrombocytopenic patients, therefore, does not necessarily indicate an increased platelet destruction rate.

Increased peripheral platelet destruction, such as seen in patients with idiopathic thrombocytopenic purpura (ITP), results in platelet life spans that are shorter than predicted by Hanson and Slichter’s model. Platelet life span studies using  $^{111}\text{In}$  are useful in discriminating between thrombocytopenia caused by decreased platelet production and that caused by increased platelet destruction. Platelet life span studies are not generally useful in the alloimmunized patient. Posttransfusion platelet counts usually provide the clinician with the necessary information for the management of these patients.

## Leukocytes

Granulocytes, lymphocytes, and monocytes—all derived from a common pluripotent stem cell—have widely variant functions and kinetics. Radionuclides have permitted the study of the normal kinetics of these various leukocyte classes.<sup>76</sup>  $^{111}\text{In}$ -oxine is now the nuclide of choice for leukocyte radiolabeling. However, because labeling with  $^{111}\text{In}$ -oxine is nonselective, the leukocytes of interest must be separated from other cells and plasma before being labeled.<sup>77</sup>

## Granulocytes

Studies conducted with labeled granulocytes have demonstrated that the circulating cells had a half-life of 3.8 to 6.7 hours. About half of the injected cells were recoverable in the circulation, with the remainder constituting a “marginating pool” of cells.<sup>78,79</sup> Labeling studies have demonstrated that the marginating granulocyte pool is about 60% of the total granulocyte pool.<sup>80</sup> Other studies have shown that labeled and transfused granulocytes transiently sequester in the lungs. The extent and duration of the pulmonary sequestration appear to be more severe when the labeled cells are suspended in saline rather than plasma.<sup>81</sup>

Alloimmunized patients demonstrate a more prolonged and intense pulmonary sequestration phase than normal subjects.<sup>82</sup> In addition, HLA and granulocyte-specific antibodies appear to reduce the intravascular half-life of transfused granulocytes<sup>83</sup> and to impair their ability to migrate to a site of active infection.<sup>84</sup>

### Lymphocytes

Lymphocytes are extremely radiosensitive, which has hampered the conduct of kinetic and imaging studies. Autologous lymphocytes labeled with a low dose of  $^{111}\text{In}$  ( $<20\mu\text{Ci}/10^8$  cells) move rapidly from the circulation and sequester in the lung, liver, and spleen, followed by a more gradual accumulation of radiolabeled cells in the lymph nodes.<sup>85</sup> These findings are consistent with studies demonstrating that lymphocytes are part of a large recirculating pool consisting of long-lived, nondividing cells.<sup>86-88</sup> Lymphocytes in this pool migrate from the circulation to the various lymphoid tissues, where they have opportunity to interact with foreign antigen. The migration patterns are modified by the lymphocyte class and the immunologic history of the cell. T cells preferentially migrate to lymph nodes, whereas B cells tend to migrate to mucosal lymphoid tissue.<sup>86</sup> Lymphocytes collected from a specific type of lymphoid tissue (eg, gut mucosa) move to that tissue because of “homing receptors” expressed on the cell after specific antigenic stimulation.<sup>89</sup>

### Activated Mononuclear Cells

There have been few kinetic studies of human monocytes because of the difficulty in obtaining a homogeneous collection of cells, and because of technical problems with cell activation and clumping during preparation. In one study, monocytes prepared by leukapheresis, density gradient separation, and countercurrent elutriation were activated with interferon- $\gamma$  and infused intraperitoneally in patients with peritoneal spread of colorectal cancer.<sup>90</sup> In two of these patients,  $^{111}\text{In}$ -labeled cells remained in the peritoneal cavity for up to 5 days after infusion, indirectly supporting the observation of clinical improvement in a larger group of patients treated with nonlabeled, activated monocytes. In another study, a subpopulation of peripheral blood lymphocytes was activated with the cytokine interleukin-2 and was found to exhibit antitumor activity. These lymphokine-activated killer cells presumably migrate to tumor sites, but cell trafficking and imaging studies have been inconclusive.<sup>91</sup> Lymphocytes directly harvested from tumor sites (tumor-infiltrating lymphocytes), however, have been shown to localize at tumor sites and persist in the circulation after activation.<sup>92</sup>

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## Development and Evaluation of Collection, Processing, and Storage Systems

With the development of new means to collect, process, and store cellular blood components comes the need to determine their clinical efficacy and the time over which they may be stored while retaining acceptable properties. The FDA has adopted a stepwise approach to this problem (Fig 21-2).<sup>93,94</sup> Relatively minor changes with a low probability of having an

impact on the recipients receiving the component are considered for approval on the basis of in-vitro biochemical and hematologic analyses. Changes that might have an effect on the ability of the cells to circulate after storage are evaluated using radiolabeled kinetic studies to document the parameters of recovery (and, in some cases, survival) in humans. Animal studies have generally not been able to be applied except to scaled prototypic systems because of volume considerations and differences in coagulation systems and cellular biochemistry. However, a severe combined immunodeficiency (SCID) mouse model for in-vivo evaluation of human platelets appears promising as an adjunct or alternative to infusion into human subjects.<sup>95</sup> Patient trials are usually required only when major alterations in the handling of the cells are being considered. The evaluation process begins at the bottom of the pyramid with the successful completion of the requirements of each phase used to qualify continuation of the evaluation higher up into the pyramid. This approach justifies the involvement of humans while, at the same time, reduces the risks they face and increases the chances for success of that phase of the evaluation. This approach also limits the use of more expensive and complex trial methods to situations where it is truly going to provide necessary information.

The FDA criteria for approval of red cell systems has evolved over the years with the intent of utilizing updated knowledge about red cell function and “raising the bar” to increase the probability that the new red cell system will provide a clinically more advantageous blood component than its predecessors, not just an equivalent one. Currently, the criteria applied to in-vitro testing (Table 21-3) state some requirements in absolute terms (that is, a particular standard must be met), while others are stated in relative terms where the analyte is compared between test and control system units. Members of this latter group are usually considered as secondary criteria where a change of more than 20% from control may be acceptable if the difference is explicable and not likely to be clinically significant. Of all these parameters, only adenosine triphosphate (ATP) levels have consistently been correlated with in-vivo recovery and, even then, primarily as a threshold: Red cells with ATP levels below about half of Day 0 values usually exhibit poor recovery.<sup>18</sup> The ATP content of red cells at the end of storage, for example, provides a better prediction of in-vivo recovery than their morphology.<sup>96</sup> Recently, the FDA has attempted to respond to concerns about the possible association between transfusion of older RBC units and poorer clinical outcomes by assessing the ability of stored red cells to use their metabolic machinery to recreate ATP and 2,3-diphosphoglycerate levels in response to incubation with a red cell rejuvenation solution. The clinical importance of this new requirement and its sensitivity in detecting changes in red cell function that are important to patients have not been determined.

The standard for recovery of radiolabeled red cells 24 hours after autologous reinfusion in healthy subjects on the last day of storage has evolved over time. Initially, 70% was regarded as

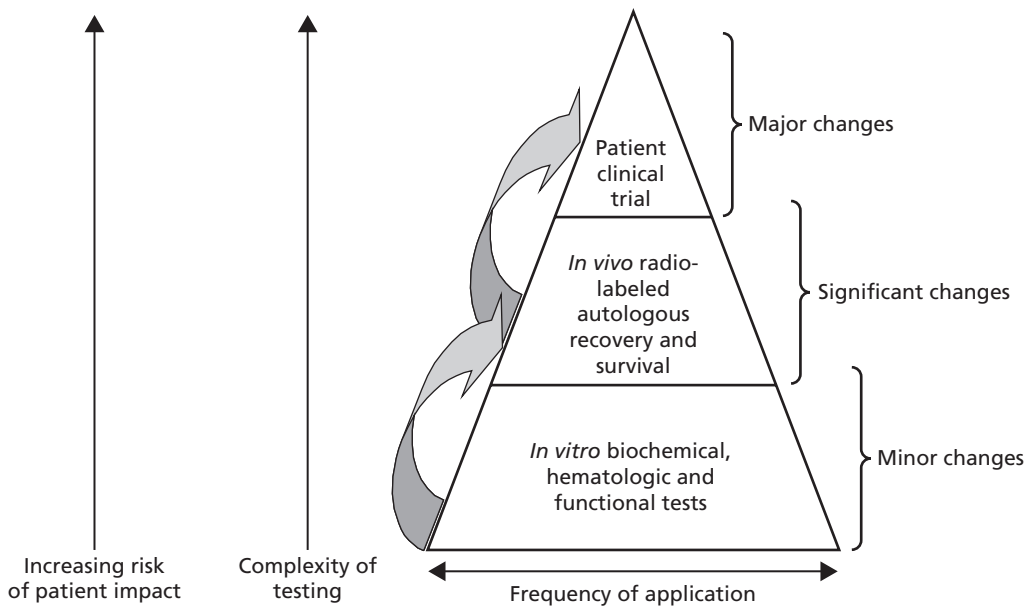


**Figure 21-2.** The progressive analysis of a new blood component illustrating 1) the stepwise nature of investigation (beginning with in-vitro testing and culminating with clinical trials) and 2) application of different degrees of scrutiny based on the extent to which the new component differed from previously investigated ones. Major changes involve extensive investigation using more complex testing techniques in response to an increased risk of adverse patient impact. Simpler changes to a collection or storage system require only in-vitro testing. Successful testing (indicative of safety and efficacy) at one level of the pyramid would be used to justify moving to the next “higher” level of testing, were that required. Reprinted with permission from AuBuchon,<sup>93</sup> adapted from a presentation of Vostal.<sup>94</sup>

**Table 21-3.** FDA Evaluation Criteria for Red Cell Systems

|                                                                                                                                                                                                                                                                                              |                                                                                                                                                                        |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Absolute Criteria</b> (Usually $\geq 60$ per condition)                                                                                                                                                                                                                                   |                                                                                                                                                                        |
| <ul style="list-style-type: none"> <li>Hemolysis <math>&lt; 1\%</math> at end of storage*</li> <li>Residual leukocytes <math>&lt; 5 \times 10^6/\text{unit}^\dagger</math></li> <li>Postfiltration red cell recovery <math>&gt; 85\%</math> of original red cell mass<sup>†</sup></li> </ul> |                                                                                                                                                                        |
| <b>Relative Criteria</b> (Allowance: $\pm 20\%$ of control population)                                                                                                                                                                                                                       |                                                                                                                                                                        |
| <ul style="list-style-type: none"> <li>ATP concentration</li> <li>2,3-DPG concentration</li> <li>Product mass</li> <li>Leukocyte concentration</li> <li>Red cell concentration</li> <li>Platelet concentration</li> </ul>                                                                    | <ul style="list-style-type: none"> <li>pH</li> <li>Mean cell volume</li> <li>Morphology score</li> <li>Lactate concentration</li> <li>Glucose concentration</li> </ul> |

\*With 95% confidence that at least 95% of the population meets or exceeds the specification.  
<sup>†</sup>Applies only to leukocyte-reduced red cell components.

adequate to avoid hemoglobinuria that could be confused with immunologic destruction of incompatible red cells. Ross and colleagues in their investigation of early candidates for RBC preservative solutions arbitrarily selected 70% as a minimum recovery.<sup>97</sup> In the early 1980s, the expectation was raised to a mean recovery of 75%. Additional expectations were later expressed that the sample mean from studies at two laboratories be at least 75% with a sample standard deviation not to exceed 9%. The most recent step in the requirements, proposed in 2004, included an additional requirement that the one-sided, lower limit of the 95% confidence interval for the proportion of the population

that would have a “successful” recovery must exceed 70%.<sup>98</sup> A “successful” individual recovery was defined as greater than 75%. This new stipulation translated into a successful trial having no more than 3 of 24 (or 2 of 20) recoveries below 75%. Studies were to be carried out at a minimum of two laboratories with a least 20 evaluable subjects. Retrospective application of the newest part of this tripartite criterion to RBC systems currently in use has suggested that defining a “successful” transfusion in this context as 67% would allow the new approach to better reflect the systems currently in wide and successful use.<sup>99</sup>

Some practical features of these evaluations have not yet been standardized. The analyses are generally easy to perform, but broad standardization of the analyses (particularly those not used in clinical practice and not subjected to periodic proficiency testing) may not always yield congruent results between laboratories involved in a trial.<sup>100</sup> Possibly even more important is the variability injected by the inherent variation between healthy subjects in both in-vitro analyses and in-vivo recovery. The most dramatic of these variations relate to inherited alterations in nucleoside metabolic pathways that result in a small proportion (perhaps 1% of normal donors) having red cells that yield poor recovery (50–60%).<sup>101,102</sup> Less striking differences are seen without demonstrable cause, and the impact of the subject’s biochemical individuality can be assessed only by using a paired control scheme in the study. Although this approach requires a study design that extends over a longer time and exposes subjects to more radioactivity, it is the only means of identifying interdonor variability as the cause of failure to meet a particular

outcome measure. In addition, a paired study design provides greater power to detect statistically significant differences between the results of test and control units. This is particularly important in the in-vivo portion of trials where the number of subjects is limited in order to reduce human radiation exposure.

## Platelets for Transfusion—Criteria for Regulatory Approval

Platelets prepared for transfusion were first described in 1963 by Freireich and colleagues.<sup>103</sup> In the intervening nearly one-half century, multiple changes and improvements have been developed and introduced into clinical medicine including: preferred storage at 22°C,<sup>104</sup> extended storage in gas permeable containers,<sup>105</sup> collection of platelets by apheresis,<sup>106</sup> platelet storage solutions,<sup>107</sup> leukocyte reduction,<sup>108</sup> and pathogen reduction.<sup>109</sup> Within each of these broad categories are multiple improvements, as competitive products have been and are continuing to be developed and introduced. Creative minds have also sought to mitigate the limited shelf life of platelets and combat the platelet storage lesion by examining metabolic or cellular signaling modulators,<sup>110</sup> freezing platelets for storage,<sup>111</sup> developing synthetic platelet-like particles,<sup>112</sup> and lyophilizing platelets.<sup>113</sup> Before any of these collection, treatment, and/or storage methods can be introduced for clinical use, the safety and efficacy must be reviewed and judged by regulatory bodies.

The FDA issued its original platelet testing guidelines in 1981 following a 1980 public workshop sponsored by the Bureau of Biologics.<sup>114</sup> These guidelines suggested a hierarchy of testing for new platelet preparations starting with a panel of in-vitro laboratory tests, followed by determination of the in-vivo recovery and survival of autologous platelets in healthy volunteers, and finally observations of the hemostatic efficacy in patients—the latter pointing to bleeding time assessments.<sup>115</sup>

Regulatory agencies have requirements for platelet content and residual contaminating cells (eg, leukocytes), which differ from country to country.<sup>116–118</sup> Generally, several in-vitro measures are required to be presented for approval to regulatory bodies. Various in-vitro tests such as platelet morphology,<sup>119</sup> extent of shape change, hypotonic shock response,<sup>120</sup> and P-selectin expression,<sup>121</sup> have been empirically correlated with in-vivo platelet recovery and survival in volunteers or corrected count increments (CCI) in patients. However, it is generally recognized that in-vitro measures are not predictive of in-vivo outcomes.<sup>122</sup> One in-vitro parameter that seems to be consistent over time as a performance indicator is the platelet pH. Yet, this is also mired in confusion because the FDA requires a pH  $\geq 6.0$  for whole-blood-derived platelets<sup>123</sup> and  $\geq 6.2$  for apheresis platelets<sup>124</sup>; AABB requires a pH  $\geq 6.2$  for both whole-blood-derived and apheresis platelets<sup>125</sup>; and the European Union requires a pH  $\geq 6.5$  and imposes an upper limit as discussed by Dumont and VandenBroeke.<sup>126–127</sup> A summary of measures applied to nascent platelet systems is provided in Table 21-4.

In-vivo kinetics of autologous platelets in healthy volunteer study subjects has been a touchstone for new and novel platelet products. These studies are generally not performed in countries other than the United States because of various regulatory issues related to the use of radiopharmaceuticals. Other countries may informally reference results from US studies in their review process, although they formally rely on in-vitro panels, CCI studies, and in-vitro predictors of hemostatic efficacy.

The understanding of platelet physiology and biochemistry as well as the development of new laboratory technologies have advanced over the decades. The development of platelet-specific monoclonal antibodies, applications of flow cytometry, analysis of cytokines, chemokine and microparticle generation, description of the platelet genome, and other potential roles of the platelet beyond hemostasis have prompted new approaches for evaluating the platelet for transfusion. Some of these measures may be added to or replace traditional in-vitro analyses. Recognizing these advances, regulatory requirements have evolved over time as new technologies for collection, storage, and secondary treatments of platelets have emerged. FDA issued a draft guidance in 1999 that expressed some of its current thinking on performance requirements for new or modified platelet preparations.<sup>128</sup> In this document, the agency delineated four categories for evaluation of platelets: in-vitro evaluation of platelet biochemistry and function, platelet survival in circulation, clinical hemostatic efficacy, and the evaluation of platelet substitutes. This document currently remains a draft document and officially is not for implementation. Even when this becomes a released guidance, it is neither binding nor obligatory. However, it does reflect the thinking of FDA, and the astute clinical protocol developer will pay close attention to it as a starting point. FDA also communicates modifications of current thinking through advisory board meetings and other communication vehicles. Thus, it is critical for those considering an approval or clearance path through FDA to have early discussions with the agency to clarify current expectations. The general categories are summarized below. These are specific to platelet function characterization. Additional requirements are prescribed that include platelet content, residual white cells, and allowable storage time. Currently there are no requirements to evaluate shipping effects on platelet preparations, although important effects from shipping have been demonstrated.<sup>129</sup>

### In-Vitro Performance Criteria

Evaluation of the in-vitro biochemistry and function of the platelet is the first step. Characterization of the platelet should be a paired comparison between a current approved or cleared storage container as a control and the test device or test process. This generally must consider the maximum time of storage for the platelet. Although not explicitly stated, it is common for FDA to expect the test outcomes to be within 20% of the control. This tolerance does not appear to be based on specific clinical outcomes. Characterization should include the platelet morphology (Kunicki scoring and/or electron microscopy) and biochemical

**Table 21-4.** Hierarchical Model for the Evaluation of Platelets for Transfusion<sup>124</sup>

| <b>In-vitro Evaluation of Platelet Biochemistry and Function</b> |                                                                                                                                                                                                                                                                                                                    |
|------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Morphology                                                       | <ul style="list-style-type: none"> <li>• Kunicki scoring and/or electron microscopy</li> </ul>                                                                                                                                                                                                                     |
| Biochemical status                                               | <ul style="list-style-type: none"> <li>• pH</li> <li>• Glucose concentrations and rate of consumption</li> <li>• Lactate concentrations and rate of production</li> <li>• ATP concentration</li> </ul>                                                                                                             |
| Activation                                                       | <ul style="list-style-type: none"> <li>• P-selectin expression</li> <li>• <math>\alpha_{IIb}\beta_3</math> activation</li> <li>• Fibrinogen binding</li> <li>• <math>\beta</math>-thromboglobulin or platelet factor 4 release</li> <li>• Platelet factor 3 activity</li> <li>• Platelet microparticles</li> </ul> |
| Response to agonists                                             | <ul style="list-style-type: none"> <li>• Agonists: ADP, collagen and/epinephrine</li> <li>• Response: aggregometry or by evaluating changes to the activation markers listed above</li> <li>• Baumgartner apparatus to assess platelet adhesiveness</li> </ul>                                                     |
| <b>Platelet Survival in Circulation</b>                          |                                                                                                                                                                                                                                                                                                                    |
| Healthy volunteer subjects                                       | <ul style="list-style-type: none"> <li>• Autologous radiolabeled platelet recovery and survival</li> </ul>                                                                                                                                                                                                         |
| Patient studies                                                  | <ul style="list-style-type: none"> <li>• Corrected count increment in transfusion-dependent patient population</li> </ul>                                                                                                                                                                                          |
| <b>Clinical Hemostatic Efficacy</b>                              |                                                                                                                                                                                                                                                                                                                    |
|                                                                  | <ul style="list-style-type: none"> <li>• Assessment of bleeding outcomes in transfusion-dependent patient population</li> </ul>                                                                                                                                                                                    |
| <b>Animal Models</b>                                             |                                                                                                                                                                                                                                                                                                                    |
|                                                                  | <ul style="list-style-type: none"> <li>• Rabbit bleeding model</li> <li>• Macaque monkey or baboon platelet recovery and survival model</li> <li>• SCID-mouse transfusion model</li> </ul>                                                                                                                         |

ATP = adenosine triphosphate; ADP = adenosine diphosphate; SCID = severe combined immunodeficiency

status (pH, glucose concentrations and rate of consumption, lactate concentrations and rate of production, and possibly ATP concentration). The platelet should be assessed for activation (P-selectin expression,  $\alpha_{IIb}\beta_3$  activation, fibrinogen binding,  $\beta$ -thromboglobulin or platelet factor 4 release, and/or platelet factor 3 activity). Platelet response to agonists such as adenosine diphosphate, collagen, and/epinephrine may be assessed using traditional aggregometry or by evaluating changes to the activation markers listed above. The Baumgartner apparatus to assess platelet adhesiveness to inverted rabbit aorta as described by Escolar is another outcome that an investigator may choose.<sup>130</sup> FDA has also suggested quantitation of platelet microparticles, although once again there is no clear clinical performance correlation between the number of microparticles in a platelet preparation and the clinical safety and efficacy. Other regulatory agencies have similar requirements with a list of various in-vitro measures necessary for approval of a novel platelet preparation.

### In-Vivo Performance Criteria—Healthy Volunteer Subjects

The second area of emphasis by FDA is platelet survival in circulation. The background, rationale, procedural, and calculation details have been published.<sup>131</sup> Although autologous radiolabeled platelet recovery and survival has been a touchstone of platelet function for decades, the BEST Collaborative, led by Dr. Scott Murphy, was concerned about the risk of having a decreasing reference for these parameters in a daisy-chain-like manner

as new preparations and treatment methods were compared to the current method over many years.<sup>69,132</sup> To avoid this “slippery slope,” a standardized control prepared from fresh whole blood was selected and evaluated.<sup>133-135</sup> The specific criteria for recovery and survival were based on historical performances and professional judgment of experts in transfusion medicine; and, lastly, on the opinion of the FDA. The requirements for this testing have been debated at length and evaluated by the BEST Collaborative. The current understanding of these requirements is that 20 to 24 evaluable subjects are to be studied in a least two laboratories. The in-vivo recovery and survival of radiolabeled autologous fresh platelets, as a control or reference, and the platelet preparation being evaluated (test platelets) are to be determined in a paired, randomized study. The aim of the study is to demonstrate that the test platelet preparation is not inferior to the fresh platelet control. The original development of this approach set the requirements that the recovery of the test platelets be at least 66% of that of fresh platelets and the survival be at least 50%.<sup>65</sup> The survival value was set lower than the recovery because the rapid clearance of platelets in thrombocytopenia renders long-term survival moot.<sup>136</sup> Current FDA expectations for recovery and survival of test platelets have evolved to them being no worse than 66% and 58%, respectively, of the fresh control. These requirements may be somewhat flexible with the agency as it weighs the risks and the benefits of a specific platelet preparation. For example, a method to inactivate pathogens in platelets adds important benefits to the unit by reducing the

patient risk of a life-threatening transfusion-transmitted infection. Therefore, the agency may be willing to accept a lower recovery and/or survival parameter when reviewing these studies.

Applying and interpreting autologous platelet kinetic studies in healthy volunteers is especially challenging for platelets prepared from multiple donors; for example, prestorage pooled platelets prepared by either the platelet-rich plasma method predominant in North America, or the buffy coat method predominant in Europe. It is generally considered an unethical practice to expose healthy study volunteers to allogeneic blood. Therefore, alternative approaches have been to study individual autologous platelet units prepared from whole blood by the applicable method, and treated as single units in secondary processes such as leukocyte reduction, pathogen reduction, and storage.<sup>65</sup> Although the results provide some information, they cannot be easily extrapolated to the final product, which is prepared from donations of four to six individual donors, ie, it is not truly the final component configuration. In these cases, the FDA and other regulatory bodies have moved to the next tier of evaluation.

### In-Vivo Performance—Patient Studies

Clinical hemostatic efficacy is the next tier of the FDA progressive requirements for platelet preparations. Depending on the degree of change associated with a new product, this tier may or may not be invoked for demonstration of efficacy. This is a judgment call by the agency often supplemented with advisory committee input. There are no universally accepted in-vivo surrogate outcomes for platelet efficacy. The first step is generally a CCI that can be determined in a transfusion-dependent patient.<sup>137,138</sup> Cutaneous bleeding time is not recognized as a sensitive or reproducible outcome that can be used as a surrogate outcome, and is generally not required.<sup>139</sup> The ultimate clinical outcome requirement is the demonstration that the novel platelet preparation is hemostatically effective when transfused to patients. These studies should contain an appropriate control arm for comparison from an approved or cleared platelet preparation method. One such trial has been recently published evaluating a pathogen reduction method.<sup>140</sup> This type of trial is expensive, complex in many facets, and lengthy to execute.

Discussion in various circles has led to the concept of an additional, fourth tier of studies in some circumstances, that of postmarket surveillance. For example, this is being considered with the introduction of pathogen-inactivated platelets as a means of providing additional verification of the safety of the new approach when applied many more times than could be included in a clinical trial. Some have recommended that hemovigilance systems be used to gather such data,<sup>141</sup> particularly when coupled with detailed, effective afferent systems to detect posttransfusion problems.<sup>142</sup>

### Platelet Substitutes

The criteria for the evaluation of platelet substitutes is quite broad and nonspecific. The ultimate requirements will be

developed by the FDA and other regulatory bodies based on the specific claim and intended clinical benefit of the product, ie, on a case-by-case basis. For example, a substitute intended for use only in acute trauma may need to demonstrate a reduction in bleeding time but may not need to have an extended circulating level in the patient—as would a product intended for prophylactic use in the marrow-suppressed patient undergoing treatment for cancer. Additional animal testing for the evaluation of prothrombotic potential, immunogenicity, and toxicity may be requested.

### Animal Models

Animal models of platelet hemostatic efficacy are not specifically required in any guidance from FDA or other regulatory body. However, these outcomes may be requested on a case-by-case basis and may be helpful in progressing through the hierarchy of proof-of-function, both from a regulatory and a general scientific query perspective. Blajchman's group has developed and published widely on the use of a rabbit bleeding model.<sup>143</sup> Thrombocytopenia is induced in the rabbit by pretreatment with gamma irradiation and platelet antisera, and the RES is blocked with ethyl palmitate. Ear bleeding time and platelet survival following infusion of study human platelet preparations is then assessed. Although some have found this model useful, it is not perfectly predictive of the response in humans. Several researchers have used murine models for evaluation of in-vivo platelet function.<sup>144</sup> There remain questions on the generalizability of murine models. In addition, it is extremely challenging to treat and store mouse platelets in a manner that is representative of how a human platelet preparation would be handled because of volume limitations. Thus, these models seem to provide some insight, but they should be interpreted with caution.

The FDA Cellular Hematology Laboratory has published their experience with a SCID mouse model for transfusion of human platelets.<sup>95</sup> They demonstrated that human platelets could be transfused to the SCID mouse and the recovery and survival kinetics described. This model is promising as an alternative to perhaps some initial human platelet kinetic studies. However, it remains to be replicated in other laboratories as well as showing a good correlation with radiolabeled recovery and survival studies in healthy subjects. Valeri and others have used the macaque monkey or baboon for evaluation of platelet preparations.<sup>145,146</sup> Again, the outcomes are useful but have not been accepted in lieu of human trials. Therefore, the complexity and expense of these studies is often a deterrent to the use of these species.

Demonstration of adequate safety and efficacy of platelets for transfusion remains a significant endeavor. Almost all tests listed above—from pH determined in the laboratory to CCI determined in patients—are surrogate measures of the ultimate clinical requirements for the transfused platelet component. Even the functional requirements of the platelet in the patient are elusive because the indications change from prophylactic use in ablative therapies, to chronic depletion in ITP, to blunt or penetrating trauma. Novel platelet products are difficult to clearly judge as

FDA, scientists, and commercial concerns all attempt to identify appropriate performance criteria. Clinical bleeding assessments are difficult to compile as consistent, precise outcomes. Heddl and others have discussed various clinical bleeding outcome methods such as days of bleeding and a recurrent event model that may find utility in these types of studies.<sup>147</sup> This remains an area where more research into appropriate clinical outcomes is needed.

The development rate of new methods does not seem to be slowing, and includes new methods to prepare components from whole blood,<sup>148</sup> pathogen reduction methods,<sup>5</sup> storage bag material changes, platelet storage solutions, novel platelet components, and evaluation of platelet substitutes.

### Specific Issues in Radiolabeling Studies

The specific procedural and methodologic details of platelet labeling studies have been examined and refined by several groups and are specifically detailed elsewhere.<sup>131</sup> Strict adherence to these methods will increase the intralaboratory precision of the technologies. Key methodologic items are the gravimetric determination of all sample volumes, determination of and correction for in-vivo radionuclide elution, and determination and correction for in-vivo baseline levels 10 to 12 days following infusion. The background and rationale for these corrections are discussed by Taylor and colleagues.<sup>23</sup> Simultaneous study of control and test platelets avoids the potential intrasubject effects that may be present over time such as a subclinical illness, described by Holme et al.<sup>35</sup>

Study design is key to permitting a clean hypothesis test and accounting for intersubject variability. Keegan et al<sup>149</sup> described the significant variability that may be observed among subjects. Although there may be various biological sources of variation between individuals such as spleen size or other aspects of the RES, some feel the most likely cause is a calculation bias imposed from poor blood volume estimates. Whatever the sources, however, the beauty of the paired design is that these effects within a subject will “cancel out” and provide a good estimate of platelet treatment effects with a smaller number of subjects than an unpaired study, as described by Dumont.<sup>150</sup> A randomized design provides further assurance of a prognostic balance between study arms.

The choice of fresh platelets prepared with a tube method as the control in studies has been the source of many animated discussions. The key advantage of this selection is the provision of a control preparation that will be consistent over time and subject neither to changes in medical device development nor variations between devices from different manufacturers. Yet, it is important to recognize that the question being asked in any given study (eg, one not leading to regulatory review) may direct that a different control be selected. For example, it may be of interest for the investigator to evaluate the difference between platelet treatment methods X and Y. Instead of comparing both X and Y to fresh, “en tube” autologous platelets, the investigator may wish to compare X and Y directly.

The analysis of platelet kinetic studies has taken many forms. The BEST Collaborative approach has specifically described a uniform method for initial data correction including adjustment for baseline levels, non-cell-bound radionuclide, and radionuclide elution in vivo. Specifically testing the difference between the two study arms by using methods such as paired t-test reduces the measurement variation and allows power to be maintained for small sample sizes. For example, studies powered on the difference in control and test can be conducted with as few as seven subjects to detect a difference of 20% recovery with 80% power with a Type I error of 0.05.

Interlaboratory variation in multicenter studies is always a concern to investigators, study sponsors, and, if applicable, regulatory reviewers. In a study evaluating the difference between 5- and 7-day stored platelets, Dumont et al<sup>151</sup> observed a 12% difference in recoveries between two laboratories. However, because the study design was paired within each subject, the interlaboratory difference did not affect the final hypothesis test. Whether this difference was because of a systematic difference in laboratory methods or subject characteristics is not known. The application of a common method between laboratories should help avoid large systematic differences. The need for laboratory proficiency evaluation as a means to qualify a laboratory to participate in this type of study has not been addressed. For the present, this is not a requirement.

### Safety

The laboratory that performs these studies must have proper procedures and practices for compliance with local, state, and national requirements for handling, dispensing, and disposal of radionuclides. These procedures safeguard users in the laboratory, the general public, and the study subjects. Procedures are generally well defined within each institution with oversight by a radiation safety committee. The welfare of the study subject is the responsibility of the principal investigator with oversight of the local institutional review board and, in the United States, the FDA. Because the use of a radionuclide as a radiopharmaceutical is approved by the FDA only for use in diagnosis and treatment in patients, use in study subjects requires FDA approval either through an investigational device exception or an investigational new drug approval.<sup>152</sup> The doses for these studies are very low (10-40  $\mu$ Ci or 0.37-1.48 MBq) and deliver an estimated total body dose of generally less than 0.24 mSv. This maximum dose is equivalent to four chest x-rays or about one-tenth of the annual exposure for a resident of the continental United States.<sup>153</sup> It is important to track total exposure of study subjects to ensure they do not exceed the annual radiation dose limit permitted, which is 5000 mrem or 50 mSv whole body dose according to the US Nuclear Regulatory Commission.<sup>154</sup> There has not been a report of any adverse events directly attributable to the use of radionuclides in studies such as this. Females of childbearing potential must be screened to ensure they are not pregnant at the time of infusion. Other potential risks to the subject participating in these studies are complications associated with venipuncture,



phlebotomy, apheresis, and infusion of blood components, such as infiltrations, infection at the venipuncture site, hypocalcemia (if anticoagulant is reinfused during the collection procedure), bacterial contamination of the blood component, fever, and chills. Such complications are rare in this setting. There may be additional concerns specific to any secondary treatment of the platelet and the teratogenicity, carcinogenicity, or mutagenicity of the product or byproducts of chemicals or reagents used in the treatment. This aspect must be investigated by the study sponsor before consideration for use in human subjects. The subject must be appropriately informed of these risks and the steps in place for their mitigation as part of the informed consent process.

### Alternatives to Radionuclides for Labeling and Determination of Platelet Kinetics

Biotin is a water-soluble vitamin also known as vitamin H or vitamin B<sub>7</sub>. Biotin is used extensively in immunologic, molecular, and biological assay systems to tag and track molecules. Biotin forms a specific and very strong interaction with avidins [dissociation constant ( $K_d$ ) is approximately  $4 \times 10^{-14}$  M for streptavidin], thus permitting highly specific detection of biotin-labeled cells in even a very dilute milieu.<sup>155</sup> As an alternative to radioisotope labeling of blood cells, biotinylated blood cells can be labeled with a streptavidin-fluorochrome conjugate and detected using flow cytometry. Biotin has been used to label red cells for estimating circulating red cell volume in infants and determining red cell recovery and survival.<sup>156,157</sup> Investigators have biotinylated platelets in several animal models, and its use for monitoring in-vivo platelet survival has been reviewed.<sup>158</sup> Ault and Knowles biotinylated murine platelets in-vivo, measured the life span of mature and reticulated platelets, and demonstrated that reticulated platelets are the youngest platelets in circulation.<sup>159</sup> Two groups have shown that two populations of platelets labeled with different biotin densities or levels per population could be simultaneously followed in the rabbit.<sup>160,161</sup> Valeri and Barnard have successfully used biotinylated platelets to measure the survival of various platelet preparations in the baboon.<sup>162,164</sup> Stahlawetz and colleagues tracked biotinylated platelets in vivo in humans to evaluate the effects of nitric oxide treatments on the platelet.<sup>165</sup>

Use of biotinylated platelets in humans has not been extensively applied, in part because of the concern for neoantigen formation in the subject. Cordle et al<sup>166</sup> observed IgG antibody formation in 3 of 20 healthy subjects exposed to biotinylated red cells. The plasma reactivity of these antibodies waned over time, and no clinical adverse events were observed. This raises the theoretical concern that there could be a cross-reactivity of these antibodies to a subject's own platelets or red cells, reminiscent of the effect observed with pegylated thrombopoietin, PEG-rHuMGDF.<sup>167</sup> For studies in neonates, there is a strong desire to avoid the use of radioisotopes, and thus investigators strongly prefer biotin labeling as the better choice to study blood volume and cell kinetics in this group of patients. Investigators using biotin in humans should follow the subjects for the formation of

biotin antibodies as a safety precaution. In addition to neoantigen formation, the presence of naturally occurring antibodies in a research subject would have unknown effects on platelet survival.<sup>168</sup> These two issues have hampered the adoption of biotin as a platelet marker.

Investigators have also used lipophilic compounds conjugated to a fluorochrome for labeling platelets. Rand compared two such compounds (PKH26-GL and PKH67-GL) to biotin-labeled platelets in the rabbit.<sup>161</sup> Michaelson used PKH2 to track the turnover of activated platelets in the baboon.<sup>169</sup> The compounds have not been used for in-vivo platelet studies in humans.

Hughes used the discordant expression of the HLA-A2 antigen on platelets to determine the survival of platelets in an allogeneic setting.<sup>170</sup> This approach, using this or other antigen systems, is intriguing for determining the kinetics of platelets in a patient without needing a specific label applied ex vivo before infusion. Further work is needed to correlate results obtained in this manner with standard, radiolabeled infusions. Allogeneic transfusions are not used in healthy volunteers for the determination of platelet survival because they raise ethical concerns related to transfusion reactions and the transmission of infectious diseases. However, use of this approach in stable patients requiring transfusions may provide a new, simple means of tracking novel platelet preparations in those for whom they are intended. The altered kinetics seen in a thrombocytopenic patient model, however, will need to be taken into account.<sup>136</sup>

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### Disclaimer

L. Dumont has disclosed relationships with Fenwal, CaridianBCT, Navigant, Haemonetics, Hemerus Medical, Terumo, Fresenius, Cerus, Immunetics, Verax Biomedical, Chiron, bioMerieux, Blood Cell Storage, and ZymeQuest. J. AuBuchon has disclosed relationships with Abbott Laboratories, Bayer, Cerus, CaridianBCT, Haemonetics, Hemerus Medical, Immunetics, Mediware Information Systems, Navigant, Pall Medical, Verax Biomedical, and ZymeQuest. R. Davey has disclosed relationships with Sangart and Johnson & Johnson.

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# II Clinical Practice

## 22

# Autoimmune Hemolytic Anemia and Paroxysmal Nocturnal Hemoglobinuria

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Autoimmune hemolytic anemia (AIHA) and paroxysmal nocturnal hemoglobinuria (PNH) represent a group of disorders characterized by accelerated red cell destruction. The first is secondary to the presence of autoantibodies directed against self-antigens on red cells. The second is characterized by increased sensitivity of red cells to complement-mediated lysis. The first description of an autoimmune hemolytic disorder was Donath and Landsteiner's description in 1904 of three cases of paroxysmal cold hemoglobinuria (PCH). Dameshek and Schwartz created the first experimental model of immune hemolytic anemia (IHA) in 1938 by inducing hemolytic anemia in guinea pigs following the injection of heterologous red cell antibodies. In 1943, Dacie and Mollison showed that patients with acquired hemolytic anemia had an intrinsic factor (presumably an antibody) that caused increased red cell destruction. The introduction of the antiglobulin test in 1945 by Coombs et al permitted the identification of the immune etiology of AIHA and established the foundation of immunohematology. Its subsequent application in patients with acquired hemolytic anemia by Boorman et al and Loutit and Mollison in 1946 firmly established the importance of red cell autoantibodies in the pathogenesis of AIHA. Subsequent refinement of the antiglobulin test permitted detection of immunoglobulin classes and complement components on the red cell membrane, and dissection of the pathophysiology of the different forms of AIHA resulting from the different immune sensitizations.<sup>1</sup>

Since PNH was first described in 1882 by Paul Strubling, several hundred cases have been reported with parallel incremental advances in knowledge of the pathophysiology and clinical course of this rare disease.<sup>2</sup> This complement-mediated hemolytic anemia has its origin in the absence of a whole class of membrane-based proteins, including those responsible for protection of cells from random complement activation. The spectrum of this disease includes aplastic anemia and leukopenia with thrombosis as the leading cause of mortality.<sup>3</sup>

## Classification of Autoimmune Hemolytic Anemias

AIHAs are divided into warm and cold autoantibody types based on the temperatures at which the antibodies maximally react with red cells in vitro. Warm autoantibodies are more reactive at 37°C than at lower temperatures, whereas cold autoantibodies react optimally at 0°C to 5°C and less strongly at higher temperatures. These two principal types are further subdivided into primary (or idiopathic) forms and secondary forms, which are associated with an underlying disorder (Table 22-1).

**Table 22-1.** Classification of Autoimmune Hemolytic Anemia

### Warm Autoantibody Type

- Idiopathic
- Secondary: lymphoma, chronic lymphocytic leukemia, hairy cell leukemia, systemic lupus erythematosus, other autoimmune disorders, ovarian tumors, chronic inflammatory disorders, following hematopoietic, cardiac, and solid organ transplantation

### Cold Autoantibody Type

- Cold hemagglutinin disease
  - Idiopathic
  - Secondary
    - Transient (acute): *Mycoplasma pneumoniae* infection, infectious mononucleosis, other infections
    - Chronic: lymphoreticular malignancies
- Paroxysmal cold hemoglobinuria
  - Idiopathic
  - Secondary: viral and other infections, syphilis

### Drug-Induced Immune Hemolytic Anemia

- Drug adsorption (hapten) type, eg, penicillin
- Drug-dependent antibody type (also referred to as immune complex), eg, second- or third-generation cephalosporins
- Autoimmune induction type, eg, α-methyl dopa
- Nonimmunologic adsorption of protein, eg, cephalothin



Lymphoproliferative disorders are present in about half of the secondary warm and cold AIHAs with systemic lupus erythematosus (SLE), antiphospholipid antibody syndrome, and other autoimmune disorders accounting for a majority of the remaining warm types. Transient, acute cold hemolytic anemias are often seen in association with infections, especially mycoplasma pneumonia and infectious mononucleosis. The proportion of warm vs cold AIHA (20% to 80%) varies widely in reported series.<sup>4</sup> This variation reflects the type of patient population studied and the particular interest and location of the investigators. As patients with assumed idiopathic etiology are observed over time, the likelihood of recognition of an associated or secondary disease increases.

A third category comprises drug-induced IHAs. The drug-induced antibodies, usually of warm type, are directed against the ingested drug or its metabolites bound to the red cell membrane or to a combined drug-red cell membrane antigen. In IHA, the antibody can target a drug or its metabolite or, as with  $\alpha$ -methyl dopa, it can induce the formation of red cell autoantibodies in a fashion analogous to classical AIHA, with hemolysis persisting even in the absence of the offending drug.

## Epidemiology

Autoimmune hemolytic anemias are relatively uncommon, with an incidence of 1 in 80,000 to 100,000 individuals. The warm autoantibody type is the most common. In a study of 347 patients,<sup>5(p29)</sup> this type accounted for 70.3%; cold agglutinin syndrome, 15.6%; PCH, 1.7%; and drug-induced IHA, 12.4%. In a study of 865 patients,<sup>6</sup> warm-type autoantibodies were found in 41%, cold-type in 32%, drug-induced in 18%, PCH in 2%, and mixed warm and cold types in 7%. Mixed AIHA with features of both warm-and cold-type autoantibodies has been found in 6% to 8% of patients in several series.<sup>7</sup> Autoimmune hemolytic anemia affects all ages, with the peak incidence of idiopathic disease in the fourth and fifth decades. Secondary AIHA reflects the age distribution of the underlying disease; lymphoproliferative disorders affect an older age group, whereas SLE involves younger patients. Women have a higher incidence of both idiopathic AIHA and secondary AIHA associated with SLE and other autoimmune disorders.

## Etiology

The development of the autoantibodies results from a breakdown of immunoregulation, possibly determined by genetic factors as seen in the inbred NZB mouse model.<sup>8</sup> The loss of suppressor T-cell regulation of autoantibody production and the presence of overactive B cells lead to the emergence of autoantibodies. Purine nucleoside analog treatment of chronic lymphocytic leukemia (CLL) is not infrequently followed by the development of AIHA via this mechanism.<sup>9</sup> Infection, inflammatory disorders, or drugs often serve as triggers to initiate this process. Viral infections also trigger the onset of AIHA by dramatically increasing the ability of macrophages to phagocytose antibody-coated erythrocytes.<sup>10</sup> Patients with warm-type AIHA sometimes have antinuclear and

antiphospholipid antibodies without clinical manifestations of SLE, or may have symptoms of hemolysis before autoimmune disease is evident. In some instances, the autoantibody is directed against another target, but cross-reacts with red cell antigens. In the cold autoantibody type occurring with mycoplasma pneumonia, cross-antigenicity between the mycoplasma cell wall and the I antigen on the red cell membrane has been suggested.<sup>11</sup> Because I antigens on cells may be receptors for attachment of mycoplasma, the mycoplasma may alter the I antigen to render it more immunogenic.<sup>12</sup> There is, however, little evidence that red cell antigens have been significantly altered to induce immune responses, because eluted antibodies react equally well with normal red cells. Eluted red cell autoantibodies also have been shown to react with the patient's red cells years later, making it unlikely that a drug, virus, or bacteria had altered the red cells during the hemolytic episodes.<sup>13</sup>

The lymphoproliferative disorders associated with AIHA represent a wide spectrum of disease, which includes CLL, Hodgkin and non-Hodgkin lymphoma, hairy cell leukemia, large granular lymphocytosis, angioimmunoblastic lymphoma, and Castleman disease. Common variable immunodeficiency may be the backdrop to AIHA and immune thrombocytopenia. Chronic infections such as subacute bacterial endocarditis and inflammatory states such as ulcerative colitis and biliary cirrhosis may be complicated by warm-type AIHA. Interferon- $\alpha$  treatment in large doses may also give rise to a warm-type AIHA. Familial cases have been reported.<sup>14</sup> More than 30 cases have been recorded in association with both benign and malignant ovarian neoplasms.<sup>15</sup> AIHA also occurs after allogeneic stem cell transplantation<sup>16</sup> as well as after solid organ transplantation<sup>17</sup>; alloimmunization following transfusion may rarely be complicated by AIHA. Infection is a common antecedent to AIHA in children.

## Warm Autoantibody Type

### Pathophysiology

The destruction of red cells occurs by intravascular, extravascular, and cell-mediated mechanisms. The reticuloendothelial system (mononuclear phagocytic system) in the spleen and, to a lesser degree, the liver is responsible for extravascular destruction of red cells. Immunoglobulin G (IgG)-coated red cells in warm-type AIHA are sequestered primarily in the spleen where vascular anatomy greatly enhances red cell interactions with macrophages. The IgG-coated red cells bind to macrophages by specific membrane receptors for the Fc portion of the IgG, subclasses IgG1 and IgG3,<sup>18</sup> and phagocytosis of the entire red cell by the macrophage sometimes follows. More commonly, there is removal of only a portion of the red cell membrane with creation of a microspherocyte released back into the circulation. These altered cells, with a decreased surface area-to-volume ratio, have a greatly shortened life span because of their loss of plasticity and increased osmotic fragility.<sup>19</sup> These cells are eventually destroyed as they obstruct flow in the microcirculation, particularly in the spleen; the presence of microspherocytes in the peripheral blood indicates ongoing hemolysis. Enhanced function of the

mononuclear-phagocytic system and an increase in Fc monocyte receptors also contribute to red cell destruction in AIHA.<sup>20</sup>

Macrophages have IgG Fc receptors only for IgG1 and IgG3<sup>21</sup>; therefore, the quantity and type of IgG on the red cell surface influences the degree of hemolysis. Red cells coated with IgG1 alone or in combination with IgG2 or IgG4 require an average of 2000 molecules of IgG per red cell to stimulate phagocytosis and rosette formation *in vitro*, whereas an average of only 230 molecules of IgG3 per red cell are required for monocyte binding.<sup>22</sup> Removal of red cells is further accelerated when complement is also present on the red cell membrane. Only IgG1 and IgG3 are efficient in activating complement. Because two IgG molecules in close proximity are required to bind C1q and activate the complement system,<sup>23</sup> there must be a sufficient number of antibody molecules and antigenic sites for complement attachment. Once C1 is bound, C4 and C2 are activated to form C3 convertase, which then cleaves C3 and C3b. Several hundred molecules of C3b are bound to the red cell membrane through the action of a single C3 convertase enzyme complex.<sup>24</sup> Macrophages have receptors for C3b and inactivated C3b (iC3b), referred to as CR1 and CR3, respectively.<sup>25</sup> The IgG Fc and complement receptors act together to enhance binding of red cells coated with both IgG and complement. Removal of IgG-coated red cells with or without complement occurs primarily in the spleen. When large amounts of IgG are present on the red cell, the liver also participates in their clearance.<sup>18</sup> The spleen functions as a “fine” filter, the liver a “coarse” filter, of sensitized red cells in AIHA.

Autoimmune hemolytic anemia has also been associated with IgA and IgM warm autoantibodies.<sup>26</sup> IgA-sensitized red cells are removed by the same splenic process as are IgG-coated red cells. Receptors for IgA have been demonstrated on mononuclear phagocytes. Intravascular hemolysis, observed in AIHA associated with IgA autoantibodies, is mediated by the binding of C5b-9 complexes to bystander red cells independent of C3 activation.<sup>27</sup> IgM autoantibodies have been shown to agglutinate or hemolyze normal red cells and enzyme-treated red cells<sup>5(p29)</sup> and to sensitize red cells with or without fixing complement. Using IgM antiglobulin serum in the direct antiglobulin test (DAT), IgM has been detected on the red cells of 8% of patients with warm AIHA, always associated with either IgG, IgA and C3, IgG and C3, or C3. With use of the more sensitive enzyme-linked antiglobulin test, IgM was found on the red cells of 38% of patients with AIHA whose DAT results were positive for IgG.<sup>28</sup> With use of the AutoAnalyzer (Bayer Diagnostics, Leverkusen, Germany), cell-bound IgM was found in 73% of patients with IgG-positive DAT results.<sup>29</sup> Several cases of AIHA with warm-reacting IgM autoantibodies in association with cell-bound C3d have been reported. In one series of children, IgM was detected on the red cells by a radioimmune assay when the DAT result was negative.<sup>30</sup> Severe hemolytic anemia, with or without the presence of IgG, has been noted in patients with warm-reactive IgM autoantibodies exhibiting specificity for sialidase-sensitive red cell antigen determinants En<sup>a</sup>, Pr, and Wt<sup>b</sup>.<sup>31,32</sup>

The quantity of the antibody on the red cell is not always an indicator of the presence or severity of hemolysis, because the interaction of several variables determines the rate of hemolysis. These include the IgG subclass, simultaneous presence of other immunoglobulin classes, affinity of the antibody, activation and presence of cell-bound complement, number of receptors on macrophages for IgG and complement, and chemical and physical characteristics of the antigens on the red cell membrane. Although there is overlap in the amount of IgG antibody on the red cells of patients with or without hemolytic anemia, patients with hemolytic anemia, on average, have more IgG molecules on their red cells.<sup>33</sup> Patients whose red cells are coated with IgA and IgM in addition to IgG are more likely to have hemolysis than those whose red cells are coated with IgG alone.<sup>34</sup> The presence or quantity of IgG3 on the red cell may not distinguish patients with hemolytic anemia from those without hemolytic anemia, but IgG3 is more commonly associated with hemolytic anemia.<sup>35</sup> The presence of autologous IgM may also determine the autoreactivity of IgG in AIHA.

### Autoantibodies and Specificity

In a report of 2000 cases of warm-type AIHA, IgG was found alone in 64% and in association with complement in 33% of cases.<sup>36</sup> IgA or monomeric IgM autoantibodies were each found in about 1% of patients with warm-type AIHA and were frequently accompanied by complement. Warm-type autoantibodies are usually polyclonal, but there can be restrictions in their Gm allotype and/or light-chain type.<sup>37,38</sup> By far the most frequent subclass of IgG warm-type autoantibody is IgG1, which is found in most patients with warm-type AIHA.<sup>36</sup> IgG1 has been found in the majority of patients with AIHA with or without hemolysis, as well as in blood donors with positive DAT results; IgG3 alone has been found in only 3% of patients and in combination with other subclasses in 5%.<sup>39</sup> IgG1 was often the only subclass identified on the red cell. IgG1 was associated with other subclasses in 37% of patients with AIHA and in 12.5% of those without AIHA. The percentage of patients with IgG3 alone was approximately the same for patients with and without AIHA, being 6.3% and 6.6%, respectively. IgG3 was found in only 2.6% of healthy blood donors and in association with other subclasses in 22% of patients with AIHA.<sup>40</sup>

Initially most red cell autoantibodies were thought to be directed to antigens of the Rh system because of weak or negative reactivity with Rh<sub>null</sub> red cells (lacking determinants of the Rh complex) and the observation that some red cell autoantibodies react preferentially with defined Rh antigens such as e, E, or C. It is now appreciated that absent or weak reactivity of autoantibodies with Rh<sub>null</sub> red cells does not always signify specificity to an Rh complex determinant, because Rh<sub>null</sub> red cells also have other membrane abnormalities, including a lack of LW and Fy, a marked decrease of U and to a lesser extent Ss, and glycophorin B levels that are 30% of normal. Eluates nonreactive with Rh<sub>null</sub> cells were shown to contain anti-LW or anti-U reactivity.<sup>41,42</sup> Many patients with AIHA have autoantibodies that

react equally well with Rh<sub>null</sub> red cells and red cells with normal phenotypes, indicating specificity to antigens outside of the Rh system. The target of these autoantibodies in selected patients include (among others) W<sup>r</sup><sub>b</sub>, En<sup>a</sup>, Ge, A, B, and antigens within the Kell and Kidd blood group systems.<sup>43</sup>

The specificity of autoantibodies in the past has been largely determined by serologic techniques. More recently, immunochemical methods have been applied to elucidate better the autoantigens or epitopes in the red cell membrane reactive with autoantibodies. In addition to Rh antigens, membrane protein band 4.1,<sup>44</sup> band 3,<sup>45</sup> and glycophorin have been identified as targets for some red cell autoantibodies.

Another source of confusion is that autoantibodies sometimes have specificity for an antigen such as E, even though the red cells from which they are eluted are negative for E.<sup>46</sup> These antibodies are most likely reacting with an antigen on the red cell that is present in larger amounts on E-positive red cells. These antibodies are termed mimicking antibodies, because they seem to be alloantibodies with anti-E specificity. Autoantibodies also may mimic alloantibodies when the corresponding antigen on the patient's red cell is weak or absent. When the patient is in remission and no longer has the serum antibody, the red cells can be shown to contain normal amounts of the antigen. Furthermore, the stored autoantibody from the patient can be shown later to react with the patient's red cells. This phenomenon has been shown to occur with Rh, LW, Kell, Duffy, Kidd, and En<sup>a</sup>.<sup>47</sup> A naturally occurring IgG autoantibody has been demonstrated in the sera of healthy persons that is specific for a senescent red cell antigen that appears as the red cell ages and is a degradation product of band 3.<sup>48</sup> A case of AIHA associated with autoantibodies to stored red cells has been reported in which the autoantibody was inhibited by a synthetic senescent red cell antigen.<sup>49</sup> Some autoantibodies have been shown to react more strongly with immature red cells than with mature red cells.

Autoantibodies have been noted after transfusion,<sup>50</sup> sometimes weeks to months after a delayed transfusion reaction. Alloimmunization has been suggested as a stimulant for autoantibody formation usually not accompanied by hemolysis.

Autoantibodies to phospholipids have been demonstrated on the red cell membrane and may account for the DAT-positive hemolytic anemia found in some patients with primary antiphospholipid syndrome or antiphospholipid syndrome secondary to SLE.<sup>51</sup> Eluates were shown to contain IgG with anticardiolipin reactivity. Antiphospholipid antibodies directed to the red cell membrane may explain the finding of IgG and complement on the red cells of some patients with SLE.

A population of IgG molecules was identified on the red cells of DAT-positive healthy blood donors that reacted with IgG red cell autoantibody but not with the red cell membrane. Radiolabeled IgG autoantibodies in eluates prepared from IgG DAT-positive blood donors and adsorbed using nonhuman and human red cells with common and rare phenotypes contained several populations of autoantibodies.<sup>52</sup> Some were bound to human red cells of common Rh phenotypes, and another, in addition to binding

to Rh red cells, also was bound to Rh<sub>null</sub> red cells. Approximately 30% of the IgG in the eluates was not adsorbed. Subsequent studies showed that some of the nonadsorbable IgG in the eluates had auto-anti-idiotypic-like reactivity in that it was able to agglutinate red cells sensitized with F(ab')<sub>2</sub> prepared from alloanti-D sera.<sup>53</sup> Further studies are required to determine whether this antibody reacts with the epitope or with Fab of the red cell autoantibody. Auto-anti-idiotypic antibodies to red cell autoantibodies have been described in NZB mice with AIHA, suggesting that the idotype-anti-idotype network affects immune regulation. The demonstration of autoidiotypic antibodies in healthy blood donors with positive DAT results and their absence in DAT-positive patients with AIHA suggest that these autoantibodies have a protective effect.

### Clinical Features

The clinical picture of warm-type AIHA is highly variable. Most patients seek treatment for symptoms attributable to anemia, such as fatigue, palpitations, and shortness of breath, but occasionally massive hemolysis manifested by hemoglobinemia, hemoglobinuria, and profound anemia is seen at onset with secondary AIHA. The associated illness often dominates the clinical picture. Autoimmune hemolytic anemia, however, may precede by months or even years the development of a disease such as SLE. Physical findings in idiopathic AIHA are related to the degree of anemia and include pallor, resting tachycardia, and mild jaundice; fever may be present in AIHA. The spleen is usually only moderately enlarged. When there is massive splenomegaly, an underlying lymphoproliferative disorder should be considered. The degree of anemia is variable and depends partly on the compensatory capacity of the marrow to respond to the increased rate of red cell destruction. Reticulocytopenia accompanying hemolytic anemia is particularly life threatening. Parvovirus infection may compound the threat of AIHA by creating red cell aplasia and absolute reticulocytopenia.

### Laboratory Features

Peripheral blood smears show microspherocytes, polychromasia, and nucleated red cells. The presence of spherocytes indicates ongoing red cell destruction. Rosettes of red cells around white cells and erythrocytes may be seen in a buffy-coat preparation; erythrophagocytosis by circulating monocytes is sometimes present. Marrow examination reveals erythroid hyperplasia, as well as increased numbers of lymphocytes or plasma cells in patients who have underlying lymphoproliferative or collagen vascular diseases. Flow cytometry of the marrow sometimes identifies a clonal population of lymphocytes even when morphologic evidence of lymphoma is not evident. The reticulocyte count is usually elevated, above the normal level of 1.5% to 2%; the red cell mean corpuscular volume is often increased to a macrocytic range. Superimposed folate deficiency can dampen or even eliminate the compensatory reticulocytosis. A rare patient will have persistent reticulocytopenia caused by inadequate marrow response or hemolytic destruction of

erythroid precursors within the marrow. Unconjugated bilirubin is usually, but not always, elevated, and urinary urobilinogen is increased. Serum haptoglobin levels are reduced or absent. With severe hemolysis, hemoglobinemia, hemoglobinuria, and urine hemosiderin can be present. Mild leukocytosis (predominantly neutrophilia) is usually seen, sometimes with thrombocytosis. Neutropenia and thrombocytopenia, however, are occasionally found. The combination of autoimmune thrombocytopenia and AIHA is referred to as Evans syndrome, usually a complication of a lymphoproliferative disorder, collagen vascular disease, or common variable immunodeficiency. If hemolysis is equivocal, conducting a red cell survival study with chromium-51-labeled red cells or measuring endogenous carbon monoxide production provides needed information. The role of the spleen in the hemolytic process can be assessed during the course of the red cell survival study by comparing isotope uptake between liver and spleen; predominantly splenic uptake anticipates a favorable response to splenectomy.

### **Antiglobulin Tests**

The diagnosis usually depends on the demonstration of a positive DAT result, indicating the presence of immunoglobulin or complement components or both on the red cell surface. A positive DAT result is often seen without hemolysis. The DAT is performed by the addition of an antiglobulin serum to washed red cells, leading to agglutination when the antibody, complement, or both are present on the red cell surface. A broad-spectrum antiglobulin reagent that contains antibodies for IgG immunoglobulins and complement components is recommended. When the test is positive, antiglobulin reagents specific for IgG or C3 are used to determine the presence of IgG or complement on the red cell. The anticomplement reagent should recognize the complement fragment C3d, which remains on the red cell surface. In special situations, antisera specific for IgA and IgM is used to demonstrate their role in the rare cases of AIHA caused by these immunoglobulin types. By the use of anti-IgG and anti-C3, three basic patterns of sensitization are observed: IgG alone, IgG plus C3, or C3 without detectable immunoglobulin. In several series of patients with warm-type AIHA, the frequency of IgG alone ranged from 18% to 64%; that of IgG plus C3, from 34% to 65%; and that of complement alone, from 10% to 33%.<sup>5(p193),36</sup>

An indirect antiglobulin test (ie, red cell antibody screen) detects free serum antibody. This test is performed by incubating a patient's serum with compatible washed donor red cells at 37°C. The donor red cells are then washed and incubated with an antiglobulin reagent to detect any serum antibodies that have adhered to the donor cells. The presence of free serum autoantibody depends on the dissociation constant of the autoantibody. It is rare to find a positive indirect test result in the absence of a positive DAT result; however, a positive DAT result is not infrequently accompanied by a negative indirect test result. With the use of enzyme-treated donor red cells, however, autoantibodies have been demonstrated in the serum of patients with AIHA

who had negative DAT results. Care must be taken in interpreting the finding of a positive indirect antiglobulin test result, because it may represent an alloantibody stimulated by previous transfusion or pregnancy, binding to transfused red cells with the target antigen. These alloantibodies must be identified by adsorption studies to ensure compatibility of red cells intended for transfusion.

### **Negative DAT Results in Patients with Warm-Type AIHA**

A small number of patients have clinical AIHA but negative DATs. They usually respond to steroid therapy, with improvement of their hemolytic anemia similar to that of patients with positive antiglobulin tests. The frequency of DAT-negative AIHA has been reported in 2% and 4% of cases, but is probably much lower when more sensitive serologic techniques for cell-bound antibody are used. The manually performed DAT requires about 300 to 500 molecules of IgG per cell, based on quantitative studies of IgG red cell autoantibodies.<sup>54</sup> Using an antibody consumption test, small amounts of IgG antibody were found on the red cells of some patients with DAT-negative hemolytic anemia.<sup>54</sup> IgG was also detected on the red cells of some patients who had the complement-alone pattern.<sup>55</sup> Using an enzyme-linked antiglobulin test and a radioimmune assay, 158 patients with AIHA had red cells with 200 or fewer molecules of IgG per cell. Two hundred molecules of IgG per cell was the threshold in this laboratory for a positive DAT result.<sup>56</sup> A significant correlation between small increases of cell-bound immunoglobulins and hemolysis was shown in the 25% of patients with evidence for immune-mediated hemolysis. Several of these patients had complement and small amounts of IgA and IgM in association with the IgG. A sensitive radioactive antiglobulin test detected IgG in four of six patients with DAT-negative AIHA.<sup>57</sup> IgG was also detected on the red cells of 13 of 20 patients whose DAT results were positive only for C3. As few as 70 molecules of IgG per red cell were measured using a radiolabeled, purified staphylococcal protein A assay.<sup>58</sup> IgG was detected on the red cells of three of nine patients with DAT-negative AIHA. A two-stage immunoradiometric assay using radiolabeled staphylococcal protein A has been described.<sup>59</sup> In this assay, red cells were initially incubated with unlabeled antiglobulin serum. If red cell autoantibodies were present, the antiglobulin antibodies would bind to the autoantibodies and be detected in the next stage with radiolabeled staphylococcal protein A. A simpler and more readily available test that does not quantitate but is capable of detecting small amounts of autoantibody below the threshold of the DAT is the Polybrene test.<sup>60</sup> Polyvalent cation hexadimethrine bromide (Polybrene) reduces the electrostatic repulsive charges on the red cells, permitting the cells to come closer together. Antibodies from one cell can then cross-link another cell, leading to agglutination that will remain after neutralization of Polybrene with a citrate resuspending solution. In a study comparing these sensitive tests, no one assay detected all patients having small quantities of IgG.<sup>61</sup> Detection is increased with a combination of tests (eg, Polybrene and an enzyme-linked antiglobulin test or flow

cytometry). Even with these sensitive techniques, an antibody sometimes cannot be detected in patients suspected of having AIHA. With the use of a gel test with anti-IgA, these DAT-negative AIHA cases are often demonstrated to be mediated by IgA autoantibodies.

#### **Positive DAT Results in Patients without Hemolytic Anemia**

The frequency of positive DAT results was 1 in 14,000 healthy blood donors in one study.<sup>62</sup> A much higher frequency of 1 in 1000 also has been reported, although many of the DATs were only weakly reactive. The subclass of IgG on the red cell in these DAT-positive healthy blood donors is mainly IgG1. IgG3 was found in only one donor. Complement alone (C3d and C4d) was found in approximately 45% of healthy blood donors. With sensitive techniques, small amounts of C3d and both C3d and C4d have been found on all normal red cells. The quantity of C3d as measured by radioimmunoassay ranged from 50 to 160 molecules per cell, which is below the amount detectable by conventional antiglobulin tests. The presence of these complement fragments on normal red cells is thought to represent continuing low-grade activation of the complement system. The DAT for complement should be performed on freshly collected EDTA-preserved blood, because the amount of C3d and C4d on the red cell increases when clotted blood is refrigerated.<sup>63</sup>

Positive DAT results for IgG are frequently found in patients receiving  $\alpha$ -methyl dopa and are usually not associated with hemolysis. C3d has been found on the red cells of children with *Plasmodium falciparum* malaria.<sup>64</sup> In two recent studies, the frequency of positive DAT results in patients with AIDS was found to be 18% and 21%, respectively.<sup>65,66</sup> In another series, 85% of patients with AIDS were found to have positive DAT results.<sup>67</sup> Red cell sensitization was attributable to varying combinations of IgG, IgM, and complement, as well as each alone. Eluates from these cells did not show anti-red-cell activity. Positive DAT results were thought to be attributable to immune complexes attached to the red cell via the CR1 receptor. In the same study, red cell CR1 receptor activity correlated inversely with a positive DAT result. Red cells from these DAT-positive patients had increased osmotic fragility and were also found in patients with SLE and circulating immune complexes, low red cell CR1 activity, and positive DAT results.<sup>68</sup> On the other hand, several cases of hemolytic anemia have been noted in AIDS and human immunodeficiency virus (HIV)-related diseases in which red cell destruction was mediated by red cell autoantibodies.<sup>69</sup> Some of these cases had reticulocytopenia despite erythroid hyperplasia of the marrow.

#### **Concomitant Warm-Type and Cold-Type AIHA**

Patients have been reported with both warm autoantibody AIHA and cold agglutinin disease.<sup>70</sup> The DAT shows both IgG and C3. The cold agglutinin titer is usually high, being greater than 1:1000 at 4°C, and the thermal amplitude of the cold agglutinin is above 30°C. Eluates from these patients' red cells contain an IgG warm-type autoantibody. In other cases of mixed-type

AIHA, the titer of IgM cold agglutinins at 4°C was less than 64, and the cold agglutinins reacted with red cells at 30°C or higher. In these patients, exposure to cold did not increase hemolysis, and treatment with glucocorticoids was effective. Hemolytic anemia tends to be severe, having a chronic course with intermittent exacerbations. In most instances, patients respond to glucocorticoids, splenectomy, or cytotoxic agents. Lymphoma and SLE are frequently associated disorders.

#### **Autoimmune Hemolytic Anemia in Children**

Autoimmune hemolytic anemia in children is either an acute, transient, self-limited disease or a prolonged chronic disorder, with the former much more common. The peak incidence is in the first 4 years of life, particularly with transient disease, which often follows well-defined viral illnesses. Patients usually recover in less than 3 months. The DAT detects only complement in the majority of these patients. Donath-Landsteiner (D-L) antibodies associated with PCH are present in some patients, but in others no antibody is found. Autoimmune hemolytic anemia of the cold hemagglutinin type is unusual. The response to glucocorticoids is rapid and constant, but their necessity may be questioned in transient self-limited cases.

Children with chronic prolonged hemolytic anemia tend to be older, and the onset is more insidious. The DAT usually shows IgG or IgG and complement. Hemolytic anemia may be intermittent or persistent for months or years and has a variable response to glucocorticoids. The hemolytic anemia is controlled in some patients by splenectomy. The overall mortality in one study of patients with chronic disease was 11.2%.<sup>71</sup>

#### **Treatment**

##### **Transfusion**

Treatment with glucocorticoids usually leads to stabilization and improvement. Because the intravascular blood volume is typically normal in these patients, the decision to transfuse is based solely on the need to increase the oxygen-carrying capacity of the blood. Transfusions should be reserved for those patients at risk because of underlying heart disease, cerebrovascular ischemia, or life-threatening anemia; care must be taken to avoid producing pulmonary edema by transfusing red cells in elderly anemic individuals whose plasma volume is already increased. Simultaneous administration of diuretics should prevent this complication. Polymerized bovine hemoglobin has been used successfully to maintain adequate oxygenation in a patient with severe AIHA; heightened research in this field is occurring to identify means of transfusion that dispense with the encumbrances of typing and crossmatching blood but no solution to this problem has yet been identified.<sup>72,73</sup>

The major problem with transfusion is the difficulty of crossmatching, because the autoantibody may result in a positive antibody screening test and an incompatible crossmatch. An alloantibody masked by the presence of the autoantibody is the major concern. Red cells from the patient should be used to adsorb the autoantibody from the patient's serum, which

can be done only in a patient who has not received a transfusion within the past 3 months. If the patient has been transfused recently, adsorption with several heterologous cells and/or a cell phenotypically matched to the patient may be performed. The adsorbed serum then can be tested for the presence of an alloantibody. The patient's red cells also should be saved for subsequent adsorptions, should further transfusions be required, and phenotyped to help identify which alloantibodies the patient could make. If an alloantibody is identified, then donor Red Blood Cell (RBC) units lacking the target antigen should be chosen for transfusion. Otherwise, the most compatible blood should be given. If the autoantibody has apparent specificity for a defined blood group antigen, then donor RBC units lacking that antigen may be chosen. However, this practice is not universal because it is recognized that even antigen-negative RBC units may be hemolyzed. It also is not practical when the autoantibody appears to have specificity for a very high-incidence antigen.

The patient is slowly given red cells in suspension and closely monitored for signs of increased hemolysis. The transfused red cells are usually destroyed as rapidly as the patient's own red cells unless the patient has responded to glucocorticoids. The notion that blood transfusion should be avoided because of the high incidence of alloimmunization and worsening of hemolysis has been refuted.<sup>74</sup> No alloantibodies related to the transfusion developed in 53 patients with decompensated AIHA who received blood, nor did any of the patients have an increased rate of hemolysis. Despite this observation, transfusion should be reserved only for those patients with life-threatening anemia; conversely, no patient should be deprived of transfusions when severe anemia complicates AIHA.

### Glucocorticoids

The patient is immediately given a short-acting glucocorticoid. The initial dose of prednisone is usually 60 mg/day, given in three divided doses, for the first 10 to 14 days. The majority of patients will have marked improvements, with decreased hemolysis and stabilization, followed by an increase in hematocrit. At this time, prednisone is consolidated to a single morning dose and reduced by 10 mg/week until a dose of 40 mg/day is reached. If clinical improvement is sustained at this dose, the prednisone is then reduced by decrements of 5 mg/week until 20 mg/day is reached. At this time, the dose can be further reduced by decrements of 2.5 mg/week, depending on the clinical response. In some patients the dose cannot be further reduced because of increasing hemolytic activity, and these patients continue to receive 15 to 20 mg/day. To reduce the side effects of glucocorticoids, an alternate-day regimen can be tried. Patients who require more than 20 mg/day to control their hemolytic anemia after 2 to 3 months of treatment should be considered for splenectomy or other treatment.

Approximately 70% of patients show improvement with prednisone treatment, with 15% to 20% going into complete remission, which allows prednisone to be discontinued. Others require less than 20 mg/day to control their disease. About 10%

of patients have little or no response to the initial high doses of prednisone. In these patients, higher doses of prednisone in the range of 80 to 100 mg/day can be tried. Another option is to use pulse steroid therapy, giving high-dose dexamethasone 40 mg for 4 days monthly.<sup>75</sup> The patient is then placed back on a maintenance level of prednisone, which is reduced according to the clinical response.

Several mechanisms have been proposed for the beneficial effect of prednisone. Its most immediate action is to decrease red cell destruction by interfering with monocyte-red cell interactions in the spleen and liver.<sup>76</sup> A later action is a reduction of the autoantibody. Prednisone also may act by decreasing the binding affinity of the autoantibody for red cell antigens.

### Splenectomy

Splenectomy is considered in a patient who either has not initially responded to high doses of glucocorticoids or subsequently requires more than 15 to 20 mg/day for control of the hemolytic disease. The decision for splenectomy should be delayed until the patient has had an adequate trial of prednisone (usually 1 to 2 months). The exception is the patient with little or no improvement when receiving high doses of prednisone (60 mg/day or more) for 2 to 3 weeks, in whom splenectomy is performed sooner. Based on a large number of reports, approximately two-thirds of patients will show a complete or partial response to splenectomy. Unfortunately, some patients relapse months or years after splenectomy. In most patients the dose of prednisone can be reduced or even discontinued after splenectomy. The response to splenectomy is significantly better in patients with idiopathic AIHA than in secondary cases; splenectomy in the latter must be recommended cautiously.<sup>77</sup>

Splenectomy removes the major site of red cell destruction in warm-type AIHA, as well as a site for antibody production (as in autoimmune thrombocytopenic purpura).<sup>78</sup> Patients may show a decrease, no change, or an increase in cell-bound antibody.<sup>79</sup> The morbidity and mortality from splenectomy are low; laparoscopic removal of spleens is now common with shortened postoperative recovery. Pneumococcal vaccine is recommended before splenectomy but also may be of value afterward.<sup>80</sup> Postsplenectomy thrombocytosis may occur and persist chronically with the complication of thrombosis; pulmonary embolism may be a cause of death in splenectomized patients with AIHA. Anticoagulation is not routinely recommended after splenectomy for patients with AIHA, but is indicated for those patients who have been shown to have antiphospholipid antibodies also.

### Rituximab

The monoclonal CD20 antibody is being used increasingly in the treatment of immune cytopenias and Evans syndrome.<sup>81</sup> This therapy was once administered only to patients who had failed splenectomy and were refractory to steroids. Because of its efficacy and excellent toleration, it is now being administered in AIHA patients before splenectomy. Its dosing at 375 mg/m<sup>2</sup> weekly for 4 to 6 weeks is the same as that used in lymphoma

regimens. In patients who respond to this intervention, maintenance therapy with smaller doses at less frequent intervals is being administered.

### Immunosuppressive Therapy

Cytotoxic drugs are also effective.<sup>82</sup> They should be reserved for patients who have not responded to glucocorticoids and splenectomy or who are poor surgical risks for splenectomy. A favorable response to a cytotoxic drug allows reduction of the steroid dose. The drug is tapered and discontinued if there has been little or no response by 4 to 6 months. The most commonly used cytotoxic agents are azathioprine (1 to 2 mg/kg/day), cyclophosphamide (1 to 2 mg/kg/day), and chlorambucil (0.1 to 0.2 mg/kg/day). Intravenous cyclophosphamide, which has been shown to be effective in the treatment of lupus nephritis, also may be of benefit in AIHA. The dosage schedule is 0.5 to 1 g/m<sup>2</sup> of body surface area given at 1- to 3-month intervals, depending on the clinical response, marrow tolerance, and leukocyte nadir (no fewer than 2000 cells/ $\mu$ L). The initial dose is 500 mg/m<sup>2</sup>. The drug is administered in 1 hour, followed by 24 hours of hydration to ensure frequent voiding to reduce bladder toxicity. White cell counts must be closely monitored. Colony-stimulating factor administration helps reverse leukopenia after chemotherapy. The long-term use of these drugs is associated with an increased risk of subsequent neoplasia. Cyclophosphamide can cause alopecia, sterility, hemorrhagic cystitis, and bladder cancer.

### Other Treatments

Other forms of treatment that have exhibited variable success include danazol, cyclosporine, mycophenolate, antilymphocyte or antithymocyte globulin, vinblastine-loaded platelets, and plasmapheresis. Cyclosporine, 4 mg/kg/day, has been used in AIHA with some success.<sup>83</sup> Patients should be monitored closely for renal toxicity. Intravenous  $\gamma$ -globulin is effective less frequently than in immune thrombocytopenic purpura.<sup>84</sup> The usual dose is 400 mg/kg/day for 5 days, followed by single doses as needed. One rationale is that blocking the Fc receptors on macrophages within the reticuloendothelial system prevents trapping of IgG-coated red cells. Another therapeutic strategy is selectively injuring macrophages by exposing them to transfused platelets sensitized with IgG platelet antibodies and loaded with vincristine.<sup>85</sup> Danazol, a synthetic androgen, also has been effective.<sup>86</sup> The initial dose is 600 to 800 mg/day, tapered to 400 mg/day once the patient has a clinical response. Side effects include mild masculinization and cholestatic jaundice. Mycophenolate has recently been reported to be successful in AIHA<sup>87,88</sup>; it is introduced in dosing of 500 mg daily and increased to 1 g once or twice daily as tolerated. Patients with persistent hemolysis should be given 1 mg of folic acid daily. Plasmapheresis is only a temporizing maneuver in the treatment of AIHA because this intervention is only weakly effective in reducing the level of IgG autoantibody in the plasma.

### Cold Autoantibody Type

Cold-type autoantibodies react most strongly with red cells at 0°C to 5°C but achieve clinical significance when their thermal

range of reactivity extends to 28°C to 31°C or higher. These are the temperatures encountered in the microvasculature of the skin, particularly in the distal extremities, ears, and the tip of the nose. Cold autoantibody types account for 15% to 25% of AIHA in most series.<sup>5(p29),36</sup> There are two basic types of cold autoantibody hemolytic anemia: cold hemagglutinin disease and PCH. Both require complement for red cell destruction and cause intravascular and extravascular hemolysis of varying degrees.<sup>89</sup>

### Cold Hemagglutinin Disease

In 1903, Landsteiner demonstrated that the red cells of an animal could be agglutinated by its sera at temperatures near 0°C. In 1926, Landsteiner and Levine showed that the sera from most healthy persons agglutinated autologous red cells at 0°C. At about this time, Iwai and Mei-Sai reported two patients with Raynaud phenomenon. In one patient, impedance of blood flow was demonstrated in vitro; in the other, sludging of blood flow, resulting from autoagglutination of red cells, was observed in the nail bed capillaries at low temperatures. In the 1950s, the association between cold autoantibodies and hemolytic anemia was established. Schuboth introduced the term "cold agglutinin disease" to distinguish this disorder from paroxysmal hemoglobinuria; the term cold hemagglutinin disease is also used.

Cold hemagglutinin disease occurs in a transient or chronic form. The transient form occurs with mycoplasmal pneumonia or infectious mononucleosis, primarily affecting adolescents or young adults. The antibody is usually IgM and polyclonal. Chronic cold hemagglutinin disease (CCHAD) usually affects persons older than 50 years of age.<sup>90</sup> The majority of these patients have no underlying illness identified; the remainder have lymphoproliferative disorders, including CLL, hairy cell leukemia, lymphomas, and Waldenström macroglobulinemia.

Although most patients with idiopathic CCHAD have IgM monoclonal cold agglutinins, a clinical picture of Waldenström macroglobulinemia or lymphoma develops in very few.<sup>91</sup> Flow cytometry may identify a monoclonal B-cell population in some of these patients without other evidence for lymphoma.

### Cold Agglutinins and Specificity

Cold agglutinins are most often reactive with the Ii blood group system. The Ii antigens are carbohydrates closely related to the ABO and Lewis blood groups and are present on red cells as glycoproteins and glycolipids.<sup>12,92</sup> At birth, cord (neonatal) red cells express large amounts of i. During the first 18 months after birth, the proportion of i antigen decreases, and the expression of I antigen increases. Most individuals predominantly express I on their mature red cells, but rarely do cells express only the i antigen. During maturation of a red cell, I antigen is formed by the action of a branching enzyme on the red cell glycoconjugates. With repeated phlebotomy, the i reactivity of red cells of healthy individuals has been shown to increase, suggesting that increased i reactivity was inversely related to the marrow transit time.<sup>93</sup> Reticulocytes express large amounts of i and reduced amounts of I. When the glycoprotein bearing the Ii antigens is extracted

from the red cell membrane, both anti-I and anti-i react with their respective antigens at 37°C.<sup>94</sup> This indicates that conformational changes in the red cell membrane induced by cold are required for the expression of the Ii antigens.

The autoantibody in patients with idiopathic CCHAD or CCHAD associated with lymphoid malignancy is usually monoclonal IgM. The specificity of monoclonal IgM with  $\kappa$ -light chains is usually directed against the I antigen,<sup>95</sup> whereas IgM cold agglutinins with  $\lambda$ -light chains are usually directed against the i antigen and only rarely against the I antigen.<sup>96</sup> Monoclonal antibodies with IgA or IgG cold agglutinins occasionally have been reported.<sup>97</sup> Cold agglutinins associated with *Mycoplasma pneumoniae* pneumonia and infectious mononucleosis are IgM and polyclonal.<sup>98</sup> Cold agglutinins associated with mycoplasmal infections are usually reactive with the I antigen,<sup>11</sup> whereas in infectious mononucleosis the IgM cold agglutinin is often reactive with the i antigen.<sup>98</sup> Approximately one-third of the cold agglutinins with anti-i specificity are also cryoprecipitable. Some patients with infectious mononucleosis and angioimmunoblastic lymphadenopathy have a cold-reacting nonagglutinating IgG anti-i and an IgM anti-IgG that both agglutinate IgG-coated red cells in the cold.<sup>99</sup>

Other specificities of cold hemagglutinins have been occasionally observed in patients with cold hemagglutinin disease. Targets for cold hemagglutinins include Pr; Gd, Sa, Lud, and Fl, which are related to Pr; Vo, which is biochemically related to Ii; and A, B, type IIH, Lewis, M-like, P, D, IA, IP, I<sup>T</sup>, I<sup>T</sup>P, and Ju.<sup>36</sup> Determination of specificity is generally not necessary in clinical practice.

### Pathophysiology

The hemolytic potential of cold agglutinins depends on their titer and ability to fix complement on red cells in vivo.<sup>100</sup> Temperatures in the superficial blood vessels of the distal extremities range from 28 to 31°C, depending on the ambient temperature. Lower temperatures occur with extremes of cold. Patients who have cold agglutinins capable of reacting with red cells in the range of 28 to 31°C will have ongoing hemolysis at ordinary room temperatures, whereas those patients with antibodies that react at a lower thermal range may have episodes of hemolysis only on cold exposure. The thermal amplitude of the antibody, which is the highest temperature at which agglutination is still visible, usually correlates with the titer of cold agglutinin. Hemolytic anemia, however, has been reported in patients with low titers of IgM cold agglutinins that have a high thermal range.<sup>101</sup>

At sites of lower temperature, IgM cold agglutinins react with red cells and bind C1. Only a single molecule of IgM is required to bind C1 and initiate the activation of the classic complement pathway.<sup>24</sup> C1 sequentially activates C4 and C2, which bind to the red cell and form a C3 convertase enzyme complex. As the blood returns to the warmer temperatures within the body, cold agglutinins dissociate from the red cell membrane, but complement activation continues. C3 convertase cleaves C3 to C3b and

C3a. A single bound C3 convertase enzyme complex can cleave several hundred molecules of C3 to C3b, many of which bind to the red cell membrane. Circulating red cells coated with C3b and iC3b are trapped predominantly in the liver, where they are bound to the CR1 and CR3 complement receptors, respectively, on the membranes of hepatic macrophages.<sup>24</sup> This interaction of red cells with macrophages results in binding, sphering, and phagocytosis of red cells. The complement sequence goes to completion on some red cells, resulting in lytic destruction and intravascular hemolysis. The human red cell is relatively resistant to complement-mediated lysis because of regulatory proteins in the serum and on the red cell membrane.<sup>24</sup>

Patients with CCHAD develop a population of red cells with a normal survival.<sup>102</sup> These resistant cells are coated with C3d that blocks further uptake of complement and develop as a result of the interaction of C3b-coated red cells with the CR1 complement receptors on macrophages. C3b is degraded to iC3b by factor I with factor H and Cr1 as cofactors. Subsequently, iC3b undergoes a second cleavage by factor I, leading to the formation of C3dg, which is further cleaved into C3d by trypsin-like enzymes. Because receptors on macrophages do not recognize these breakdown products of C3b, the red cells are released from the liver. A substantial number of C3b-coated red cells initially cleared by the liver are released back into the circulation as C3d-coated red cells by this mechanism.<sup>18,103</sup>

Hemolysis secondary to cold agglutinin disease may also be exaggerated with any acute phase reaction that leads to an increase in complement production; infection frequently precipitates increased hemolysis in CCHAD.<sup>104</sup>

### Clinical Features

Patients with CCHAD often present with symptoms of chronic anemia. Episodes of acute hemolysis occur after cold exposure, manifested by hemoglobinemia and hemoglobinuria. Patients may note acrocyanosis of their distal extremities, nose, ears, and chin on cold exposure. Livedo reticularis, a sky-blue mottling of the skin of the extremities, is a result of agglutinated red cells impeding blood flow in the capillary bed. Some patients experience Raynaud phenomenon. Rarely, a patient develops a vascular occlusion, usually of a distal extremity, which is precipitated by prolonged exposure to cold. Patients with IgA monoclonal cold agglutinins manifest acrocyanosis but no hemolysis, because IgA does not activate the classic complement pathway. The course of patients with idiopathic CCHAD is characterized by either chronic anemia or episodes of hemolysis, depending on the thermal amplitude of the cold agglutinin. In a few patients this progresses to a malignant lymphoproliferative disease. Features of malignant lymphoma may overshadow cold agglutinin disease. A very large spleen should suggest malignant lymphoma; flow cytometry may demonstrate a monoclonal B-cell population with the light-chain restriction of the circulating IgM agglutinin.

In acute transient cold agglutinin disease, most often associated with *M. pneumoniae* pneumonia or infectious mononucleosis,



hemolytic anemia appears in the second or third week of the illness; this interval between the acute illness and the subsequent bout of hemolysis leads to significant under-recognition of this phenomenon. Cold agglutinin disease rarely has been observed in association with chickenpox and infectious diseases other than the ones noted.<sup>105</sup> The hemolysis in these disorders can be severe, resulting in significant hemoglobinuria and transient renal failure. Hemolytic anemia usually subsides spontaneously in 1 to 2 weeks, with the hematocrit quickly returning to normal. Patients may experience acrocyanosis, especially in a cold room. The spleen may be modestly enlarged.

Laboratory Features

The first indication that a patient might have cold agglutinins sometimes comes from the laboratory or blood bank after the observation of autoagglutination of the patient's red cells at room temperature. Blood smears show polychromasia, agglutinated red cells and, at times, spherocytes. The reticulocyte count and index are usually elevated. Clumping of red cells may lead to errors in calculating red cell indices because of autoagglutination. An elevated mean corpuscular volume that corrects to normal on heating the blood sample is good evidence for a cold agglutinin. Slight increases in unconjugated bilirubin and hemoglobinuria are sometimes present, especially after hemolytic episodes. Urine hemosiderin is often present.

Antiglobulin Tests

The DAT in patients with cold agglutinin disease shows C3 and, more specifically, C3d. When the DAT is performed, the red cells should be washed thoroughly at 37°C, because a small amount of cold agglutinin will interfere with the test. When cold agglutinins are being assayed, the red cells are separated from serum or plasma at 37°C so that cold agglutinin does not remain adsorbed to the red cell membrane. Normal donor cells warmed to 37°C are added to dilutions of the patient's plasma, which is also prewarmed to 37°C. Tubes are incubated at 37°C, then incubated at 4°C for at least 30 minutes to 1 hour. Agglutination occurring at cold temperatures should be reversible at 37°C. Healthy persons have low titers of IgM cold agglutinins that usually do not exceed a titer of 1:64 at 4°C. The specificity of these cold agglutinins is almost always anti-I.

Patients with pathologic cold agglutinins usually have titers at 4°C of 1:1000 or more in saline.<sup>5(pp213-23)</sup> When the cold agglutinin titer is less than 1:1000, a thermal amplitude test can be performed. The patient's serum is initially tested against normal cells suspended in saline at 20°C. If negative, it is unlikely that cold agglutinins are causing the hemolytic anemia. If positive, a test is performed at 30°C in albumin; a positive test indicates that the cold agglutinin is of clinical importance (Table 22-2).

Treatment

In most patients with CCHAD, anemia is mild and treatment is largely symptomatic. Patients are advised to keep warm (particularly extremities). Large amounts of ice-cold beverages should

Table 22-2. Autoimmune Hemolytic Anemia

| Type                           | DAT  | Serum Antibody                                                                                                             | Antibody Specificity                                                                                         |
|--------------------------------|------|----------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------|
| Warm autoantibody type         | IgG  | Serum antibody in <50% with untreated red cells                                                                            | Rh complex antigens in the majority; others include LW, U, W <sup>r</sup> , En <sup>a</sup> , Kidd, Kell, Ge |
|                                | IgG+ |                                                                                                                            |                                                                                                              |
|                                | C3d  |                                                                                                                            |                                                                                                              |
|                                | C3d* |                                                                                                                            |                                                                                                              |
| Cold autoagglutinin type       | C3d  | Pathologic cold agglutinins<br>a. 1:1000 at 4°C<br>b. Positive at 30°C in albumin                                          | Mainly I, also i and Pr                                                                                      |
| Paroxysmal cold hemoglobinuria | C3d  | Biphasic hemolysin antibody reacts with red cell in cold; hemolysis occurs at 37°C in presence of complement (fresh serum) |                                                                                                              |

\*IgG can be detected on red cells apparently coated with C3d alone by more sensitive techniques; warm-reacting IgM antibodies also may fix complement to red cells.

DAT = direct antiglobulin test; IgG = immunoglobulin G; IgM = immunoglobulin M.

be avoided. Because of the increased red cell turnover, folic acid (1 mg/day) should be given. In patients with more severe hemolytic anemia, chlorambucil or cyclophosphamide is given, and in some patients this results in decreased titers of cold agglutinins and reduced hemolysis.<sup>106</sup> Glucocorticoids and splenectomy generally are not effective, with some important exceptions. Prednisone was shown to be beneficial in patients with low titers of IgM cold agglutinins having high thermal amplitudes and in patients with IgG cold agglutinins.<sup>107</sup> In the latter group, splenectomy was also effective. Plasmapheresis may provide temporary amelioration of hemolysis.<sup>108</sup> α-Interferon therapy has been given to patients with severe cold agglutinin disease with variable results.<sup>109</sup> CD20 monoclonal antibodies have been successful in treatment of some patients with cold agglutinin disease<sup>110,111</sup>; flow cytometry of the marrow may identify a target for this effective and relatively innocuous immunotherapy.

Transfusion should be reserved for patients whose cardiovascular or cerebrovascular systems are significantly compromised by the degree of anemia. Washed RBC units are preferable, to avoid supplying additional complement components to a patient whose low complement levels may be limiting the rate of hemolysis. The donor red cells should be infused through a blood warmer, and the patient should be kept warm during the transfusion. If compatibility testing is performed using prewarmed techniques, difficulties are usually not encountered.

Because acute cold agglutinin disease associated with infection is self-limited, the best treatment is supportive therapy and judicious inaction. If the hemolysis is severe, these patients

should be well hydrated to maintain renal blood flow. Acute renal failure occasionally occurs in the setting of severe hemolysis.

### Paroxysmal Cold Hemoglobinuria

A rare form of AIHA, PCH is characterized by the sudden onset of severe hemolysis particularly after infection. Children are most commonly affected. The disorder was first characterized by Donath and Landsteiner in 1904. They demonstrated the bithermic nature of this hemolysis, which they attributed to an initial sensitizing cold-reacting autohemolysin and a subsequent lytic warm-reacting serum factor. The biphasic laboratory test for PCH carries their names. Originally, PCH was most often associated with congenital syphilis and appeared as a chronic disorder with acute episodes of hemolysis precipitated by cold exposure, hence the term “paroxysmal cold hemoglobinuria.” Today, PCH most often is encountered as an acute transient hemolytic anemia in children after a variety of infections with little if any cold exposure.<sup>112</sup>

### Pathophysiology

The D-L autohemolysin is an IgG autoantibody that reacts with red cells at reduced temperatures in the extremities and fixes the earlier components of complement (see Cold Hemagglutinin Disease). As blood returns to warmer temperatures within the body, complement activation continues. Some cells are coated with C3b and cleared by the liver and spleen, whereas others are lysed by the terminal components of complement.

The D-L antibody in patients with the acute transient form of the disease seldom binds to the red cell in vitro at temperatures higher than 20°C. However, hemolysis occurs in these patients even when they are not exposed to the cold, indicating that the D-L antibody is able to react with red cells at higher temperatures in vivo. The explanation for this paradox is not known. Patients with chronic PCH usually experience hemolysis only when exposed to the cold, similar to patients with CCHAD.

In most patients the D-L antibody is IgG with anti-P specificity.<sup>113</sup> The P antigen is a globoside. The D-L antibodies are inhibited by globoside and Forssman glycolipids, which are widespread in microorganisms.<sup>114</sup> The D-L antibody, therefore, may represent an immunologic response to cross-reacting antigens present in microorganisms.

Paroxysmal cold hemoglobinuria is reported to be associated with a wide variety of infections, including measles (and measles vaccination), mumps, cytomegalovirus, infectious mononucleosis, chickenpox, and mycoplasmal pneumonia, as well as *Haemophilus influenzae*, *Klebsiella pneumoniae*, and *Escherichia coli* infections. Frequently, the cause of the preceding illness is not identified.

### Clinical Features

An acute attack typically occurs during the time a patient is recovering from a recent upper respiratory tract infection. Attacks are characterized by the sudden onset of shaking chills,

back and leg pain, and abdominal cramps. Fever often follows and can be as high as 40°C. Fresh urine passed after the onset of symptoms usually contains hemoglobin. The attack may not be associated with any obvious cold exposure. The constitutional symptoms usually subside within a few hours. Cold urticaria may accompany an attack.

Attacks of PCH can be severe and life threatening. Most patients recover in a few days to several weeks without recurrence. Transient renal failure secondary to hemolysis develops in an occasional patient.

### Laboratory Features

The hemoglobin level can be quite low. Reticulocytopenia can occur initially, but reticulocytosis subsequently develops. Leukopenia is followed by leukocytosis. Unconjugated bilirubin is increased. Hemoglobinemia and hemoglobinuria can be present. Serum complement levels are usually depressed during an acute attack. Peripheral blood smears show autoagglutination of red cells, polychromasia, nucleated red cells, spherocytes, and erythrophagocytosis involving monocytes or neutrophils or both.

The DAT result is positive for C3d. The D-L antibody is detected by the biphasic D-L test. The patient's serum is mixed with donor red cells and fresh normal serum as a source of complement. The mixture is incubated at 4°C, then warmed to 37°C. Hemolysis will occur when the D-L antibody is present. In some instances, the sera of patients with cold agglutinin disease may give false-positive results when the cold agglutinin has the property of a monophasic cold hemolysin. The frequency of false-positive results is greatly increased if enzyme-treated cells are used in the D-L test, making a control for monophasic lysis essential. False-positive results appear in only 2% of cold agglutinin sera when untreated red cells were used in the D-L test.<sup>115</sup> A low titer of the D-L antibody may be present for several weeks to months after an episode of PCH (Table 22-2).

### Treatment

Paroxysmal cold hemoglobinuria is usually a self-limiting illness, with recovery occurring in a few days or a week. Patients are usually treated symptomatically by being kept warm. When transfusion is needed, P-positive red cells can be given and are unlikely to precipitate hemolysis, provided the blood is administered through a warmer.<sup>113</sup> Glucocorticoids and splenectomy are usually not effective. Glucocorticoids are often given empirically and, when given, should be tapered as quickly as possible. Although syphilis is rarely a cause today, it should be excluded in patients with chronic PCH. Patients with chronic idiopathic PCH should be advised to avoid cold and usually do not require any specific treatment.

### Drug-Induced Immune Hemolytic Anemia

Several drugs can produce a positive DAT result and, in some patients, hemolytic anemia. Drug-induced IHA has been reported in 12.4% and 18% of patients with AIHA.<sup>5(p29)</sup> The first description of drug-induced, immune-mediated cytopenia was

by Ackroyd<sup>116</sup> in 1949, who reported thrombocytopenic purpura after allylisopropylacetyl carbamide administration. In 1953, Snapper et al<sup>117</sup> described a patient with pancytopenia, hemolytic anemia, and a positive DAT result who was receiving mephenytoin. In 1956, Harris<sup>118</sup> reported hemolytic anemia recurring in patients after they received a second course of stibophen used in the treatment of schistosomiasis.

Three mechanisms by which drugs induce IHA have been described<sup>5(pp267-304)</sup>; drug adsorption (hapten), immune complex (innocent bystander), and induction of autoantibody. A fourth mechanism, nonimmunologic adsorption of proteins to the red cells, produces a positive DAT result but not hemolytic anemia. The so-called immune complex mechanism, which has accounted for the majority of reported cases of drug-induced IHA, is presently thought in most instances not to be mediated by circulating drug-antidrug immune complexes that bind non-specifically to red cells, but by a specific interaction of a drug, the antibody, and red cells.

Evidence for this drug-dependent mechanism is provided by studies<sup>119</sup> showing that quinidine-induced antibodies in the presence of quinidine were bound to the platelet through the Fab region or antigen-binding site and not through the Fc region of the antibody, as would be expected for binding of immune complexes. In studies on tolmetin-dependent, antibody-associated hemolytic anemia, the drug antibody in the presence of the drug was bound to the red cell through the Fab region.<sup>120</sup> Drug-dependent antibodies react with a certain cell line (red cell, platelet, or white cell) and may recognize well-defined specific cell membrane antigens.<sup>121</sup>

Types of drug-induced IHA discussed below are 1) a drug adsorption mechanism, 2) a drug-dependent antibody mechanism, 3) an autoimmune induction mechanism, and 4) a nonimmunologic adsorption of protein (Table 22-3). The

situation may be more complicated because some drugs may create hemolytic anemia via multiple mechanisms simultaneously in the same patient; diagnostic tests must be pursued with this in mind.

Drug Adsorption Mechanism

In the drug adsorption mechanism, drug-induced antibodies react with a drug already firmly bound to the red cell membrane. Penicillin is a prototype of drugs that produce IHA by this mechanism.<sup>122</sup> Penicillin is covalently bound to red cell membrane protein and is not removed by washing the cells. Penicillin can be demonstrated on the red cells of 30% of patients receiving 1.2 to 2.5 million U/day and of all patients receiving 10 million U/day.<sup>122</sup> Penicillin on these red cells does no harm if immune sensitization does not occur. Approximately 3% of patients receiving massive doses of intravenous penicillin in the range of 10 million U/day continuously for several days eventually will have a positive antiglobulin test result, but hemolytic anemia is rare. For hemolytic anemia to develop, intravenous doses of 10 million units or more over several days are necessary. Although IgM antibodies directed against the benzylpenicilloyl determinant of penicillin develop in approximately 90% of patients who receive penicillin, these antibodies are usually not involved in the pathogenesis of hemolytic anemia.<sup>123</sup> The antibody involved is the IgG directed against the benzylpenicilloyl determinant or other metabolites of penicillin. The onset of hemolytic anemia is usually after 7 days of treatment but may be sooner in patients who have been previously immunized to penicillin. The mechanism of red cell damage is largely extravascular, occurring mainly in the spleen. The degree of hemolysis is usually mild to moderate.

The DAT shows IgG or occasionally IgG and complement.<sup>5(pp267-304)</sup> An indirect antiglobulin test result is negative unless donor cells coated with penicillin are used. Similarly, eluates from the patient's red cells react only with penicillin-coated donor red cells. The finding of a positive DAT result by itself does not dictate discontinuing the drug unless there is evidence for hemolysis. Once the drug is stopped, the hemolytic anemia abates within a few days. Prednisone is usually not required to control the hemolytic anemia, because the disease is self-limited. Penicillin antibodies can cross-react with cephalothin-treated red cells, and antibodies against cephalothin can cross-react with penicillin-coated red cells.<sup>124</sup> Although cephalothin also binds strongly to the red cell membrane, it rarely causes hemolytic anemia. Other drugs that induce hemolytic anemia by this mechanism include tolbutamide, tetracycline, and streptomycin.<sup>125</sup>

Drug-Dependent Antibody Mechanism

In this mechanism (formerly termed immune complex/innocent bystander mechanism), a drug is thought to bind transiently with proteins on the red cell membrane to form an immunogen that stimulates production of an antibody.<sup>126</sup> The demonstration of the antibody in vitro requires the presence of a free drug or its metabolites on red cells. For some drug-dependent antibodies, reactivity occurs only with red cells of a specific blood group.

Table 22-3. Drug-Induced Immune Hemolytic Anemia

| Type                                         | DAT         | Serum Antibody                                                                                                      | Hematologic Manifestations                                              |
|----------------------------------------------|-------------|---------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------|
| Drug adsorption (eg, penicillin)             | IgG<br>C3d* | Antibody reacts with drug-coated; eluates react only with drug-coated red cells                                     | Moderate degree of hemolysis, usually extravascular mechanism red cells |
| Drug-dependent antibody type (eg, cefotetan) | C3d         | Ab + drug + red cell → sensitization, agglutination or hemolysis of red cells; Ab is IgG or IgM; eluate is negative | Abrupt onset of severe intravascular hemolysis; renal failure           |
| Autoimmune induction (eg, α-methyl dopa)     | IgG         | Autoantibodies against red cell; eluate reacts with red cell                                                        | Mild-to-moderate degree of extravascular type hemolysis                 |

\*Present in approximately 40% of penicillin-induced immune hemolytic anemia. DAT = direct antiglobulin test; IgG = immunoglobulin G; IgM = immunoglobulin M.

In previously sensitized patients, only a small quantity of the drug is required to initiate hemolysis. Hemolysis is abrupt and can be severe, causing massive hemoglobinemia and hemoglobinuria. Renal failure develops in approximately one-third of patients.<sup>5(p29)</sup> Other complications include shock and disseminated intravascular coagulation.

All drugs should be discontinued until the responsible drug can be determined in the laboratory. Hemolysis usually disappears within a few hours or days. Patients should be observed closely for renal failure. The drug-dependent antibodies can persist for years, placing patients at great risk if they are exposed again to the responsible drug. Drug-induced autoantibodies, which also may be present, rapidly disappear within a few days after drug administration is stopped.

The responsible antibody may be IgG or IgM and is capable of activating complement. The DAT shows only C3d. In many cases the responsible drug is recognized by the antibody only when it is associated with a specific blood group antigen. In such cases, the drug and its antibody will not react with red cells lacking the specific antigen. Drug-dependent antibodies associated with rifampicin, nitrofurantoin, and dexchlorpheniramine have been shown to react with cells rich in I antigen.<sup>127,128</sup> Drug-dependent antibodies associated with thiopental and nomifensine are reactive with adult, but not cord, red cells.<sup>129</sup> Drug-dependent antibodies in a patient with chlorpropamide-induced hemolytic anemia were detected only with Jk<sup>a</sup>-positive red cells.<sup>130</sup> In some patients with drug-dependent IHA caused by nomifensine, the serum did not react with red cells in the presence of the parent drug but did react with red cells in the presence of urine, which contained the ex-vivo antigen-metabolite.<sup>131</sup> An ex-vivo antigen-metabolite was also required to detect drug-dependent antibodies in a case of IHA caused by diclofenac.<sup>132</sup> In cases of suspected drug-dependent IHA that are negative with the parent drug, it is important to test with ex-vivo antigens-metabolites present in urine or serum collected from volunteers or patients after ingestion of the drug.<sup>133</sup>

Many drugs have been reported to cause drug-dependent IHA; examples include nonsteroidal anti-inflammatory drugs (NSAIDs) and second- and third-generation cephalosporins.<sup>134,135</sup> In a patient receiving cefotaxime, severe intravascular hemolysis developed, which showed in-vitro characteristics of drug-dependent and drug adsorption mechanisms.<sup>136</sup> In a patient treated with cefotetan in whom hemolytic anemia and fatal renal failure developed, the serum contained antibodies that reacted with cefotetan-treated red cells, antibodies that reacted only with untreated red cells in the presence of the drug, and drug-independent autoantibodies.<sup>137</sup> Both drug adsorption and drug-dependent antibodies were associated with ceftazidime-induced IHA.<sup>138</sup> A case of sulindac-induced IHA was associated with a drug-dependent antibody having no blood group specificity.<sup>139</sup> A patient receiving both suprofen and tolmetin (NSAIDs) had severe intravascular hemolysis and renal failure. Drug-dependent antibodies to both were discovered; autoantibodies also were present.<sup>140</sup> Phenacetin<sup>141</sup> and carbimazole<sup>142</sup>

also have been associated with drug-dependent antibodies and autoantibodies.

### Autoimmune Induction Mechanism

$\alpha$ -Methyldopa induces a positive antiglobulin test result in 11% to 36% of patients after 3 to 6 months of therapy.<sup>143</sup> The incidence of a positive DAT result seems to correlate with the drug dose. The DAT results were positive in 11% of patients taking 1 g/day or less, 19% in those taking 1 to 2 g/day, and 36% in those taking more than 2 g/day.<sup>143</sup> However, hemolytic anemia develops in less than 1%. On second exposure to the drug, the interval for the development of a positive DAT result is again 3 to 6 months, thus differing from the usual anamnestic immune response.

The positive DAT result shows IgG and occasionally IgG and complement.<sup>5(pp267-304)</sup> The indirect antiglobulin test is also positive, especially in those patients with hemolytic anemia. Eluates from patients' red cells as well as serum antibody do not require the drug to react with the red cell. The antibody specificity is to antigens related to the Rh complex.<sup>144</sup> This conclusion is supported by the observation of Bakemeier et al<sup>144</sup> that antibodies associated with  $\alpha$ -methyldopa AIHA recognize a 34-kD polypeptide and a 37- to 55-kD glycoprotein, which seem to be antigens of the Rh family.

The mechanism by which  $\alpha$ -methyldopa induces AIHA is unknown. It has been proposed that  $\alpha$ -methyldopa alters the red cell membrane to form neoantigens recognized as foreign, but there is little evidence to support this concept.  $\alpha$ -Methyldopa causes an increase in lymphocyte cyclic adenosine monophosphate, which may inhibit suppressor T cells<sup>145</sup> and lead to overproduction of red cell autoantibodies. However, Garratty et al<sup>146</sup> were unable to confirm  $\alpha$ -methyldopa depression of suppressor cell function.  $\alpha$ -Methyldopa-induced red cell autoantibodies rarely produce hemolytic anemia. Although impaired reticuloendothelial function has been demonstrated in patients receiving  $\alpha$ -methyldopa,<sup>147</sup> this cannot by itself explain the infrequency of immune hemolysis in these patients.

The degree of hemolysis is usually mild to moderate, and anemia develops gradually. Hemolysis usually regresses within a few days after the drug is stopped but may continue for several weeks to months. Generally no treatment is necessary. Glucocorticoids may shorten the period of recovery but are usually not required. A positive DAT result is not by itself an indication to discontinue  $\alpha$ -methyldopa unless there is evidence of hemolytic anemia. The DAT result may remain positive for months to years after discontinuation of  $\alpha$ -methyldopa. Other drugs that may induce hemolytic anemia by this mechanism include levodopa,<sup>148</sup> mefenamic acid,<sup>149</sup> and procainamide.<sup>150</sup>

### Nonimmunologic Adsorption of Protein

A small number (5%) of patients receiving cephalothin eventually have positive antiglobulin test results because of nonspecific adsorption of serum proteins.<sup>5(pp267-304)</sup> The DAT result becomes positive after a few days of treatment. Albumin, fibrinogen, complement, and immunoglobulins are detected on the red cell

membrane.<sup>151</sup> As noted earlier, cephalothin can produce hemolytic anemia by the drug adsorption mechanism, but this is unusual.<sup>152</sup>

Red cells treated in vitro with the chemotherapeutic agent cisplatin can adsorb IgG nonimmunologically and produce a positive DAT result.<sup>153</sup> Several cases of hemolytic anemia associated with cisplatin treatment have been reported, but in only one were antibodies to cisplatin demonstrated.<sup>154</sup> Conceivably, nonimmunologic binding of IgG was responsible for the positive DAT results, whereas other unknown mechanisms may have caused that anemia.

Paroxysmal Nocturnal Hemoglobinuria

Paroxysmal nocturnal hemoglobinuria is a rare acquired hemolytic anemia with protean manifestations that have their shared origin in the absence of an essential anchoring protein for many cell membrane-based molecules on the surface of hematopoietic elements.

Clinical Features and Biology

Clinical Picture and Course

Mild to moderate anemia is the most frequent initial clinical sign (Table 22-4). Complement-mediated intravascular hemolysis and hemoglobinuria can create the double insult of iron deficiency and hemolytic anemia. Passage of dark, hemoglobin-containing urine upon arising is the initial symptom in only a minority of cases. Chronic low-grade hemolysis is punctuated by hemolytic crises that are precipitated by infection, drug administration, transfusions, and other stresses.<sup>155</sup> Adults 25 to 45 years of age are most frequently affected, although no age group is free of its involvement. Physical findings are related to anemia; some patients have mild splenomegaly.<sup>156</sup> The course of the disease varies greatly, with 50% of patients surviving more than 15 years<sup>155</sup>; interventions such as marrow transplantation now offer the possibility for cure

Table 22-4. Clinical Features and Diagnosis of Paroxysmal Nocturnal Hemoglobinuria

| Features            | Clinical Manifestations                                                                                                   |
|---------------------|---------------------------------------------------------------------------------------------------------------------------|
| Signs and symptoms  | Anemia, hemoglobinuria, thrombosis, infection                                                                             |
| Age at onset        | 25–45 years                                                                                                               |
| Laboratory findings | Moderate pancytopenia; elevated reticulocyte count, lactic dehydrogenase, and bilirubin; hemosiderinuria; iron deficiency |
| Confirmatory tests  | Flow cytometry for DAF and MIRL; positive acidified serum and sucrose hemolysis tests                                     |
| Survival            | Averages >10 years from diagnosis with cure possible with marrow transplantation                                          |
| Complications       | Venous thrombosis (especially hepatic vein); infection; aplastic anemia; acute leukemia                                   |

DAF = delay accelerating factor; MIRL = membrane inhibitor reactive lysis.

of the disorder. Fatalities most frequently result from thrombotic and infectious complications. Hepatic and portal veins are often the sites of atypical thrombosis in this disorder.<sup>157</sup> Smooth muscle dystonias with esophageal spasm, erectile dysfunction, and pulmonary hypertension all have their origins in lowering of nitric oxide levels secondary to its binding to free hemoglobin. Paroxysmal nocturnal hemoglobinuria often occurs against the backdrop of aplastic anemia with this relationship contributing to theories regarding the etiology of the disorder.<sup>158</sup> It may also evolve into aplastic anemia or acute nonlymphocytic leukemia.<sup>159</sup>

Etiology

Paroxysmal nocturnal hemoglobinuria results from a somatic mutation in the X-linked phosphatidylinositol glycan (PIG)-A gene in a hematopoietic stem cell line; more than 100 mutations in the gene have been described with the most common a “frameshift” mutation.<sup>160</sup> The PIG-A gene product contributes to the construction of the glycosylphosphatidylinositol (GPI) anchor protein, which constitutes the cytosolic tail of numerous transmembrane cell surface proteins. Almost 100 different GPI-anchored proteins have been identified, helping to explain the many manifestations of PNH. The deficit in two GPI-based molecules [decay-accelerating factor (CD55 or DAF) and membrane inhibitor of reactive lysis (CD59 or MIRL)] results in an inability to inactivate complement on the red cell surface, leading to hemolysis. A deficiency of a urokinase plasminogen activator receptor on the surface of monocytes has been suggested as contributing to the thrombophilic complications of PNH.<sup>155</sup>

The appearance and persistence of the PNH clone(s) in aplastic anemia has suggested that the PIG-A– cells possess a selective advantage over PIG-A+ cells. The correlation of PNH with immunologically mediated aplastic anemia supports the hypothesis that the altered PNH clone escapes an immune-mediated assault, leading to aplastic anemia.<sup>161</sup> An interaction between certain GPI-anchored proteins and the immune system may be crucial for PNH pathogenesis. Affected cells may have a survival advantage after some injury sustained by the normal progenitors, consistent with the hypothesis of immune escape.<sup>162,163</sup>

Pathogenesis

The increased sensitivity to complement (C′) of blood cells from patients with PNH is the result of the PIG-A membrane defect, and the C′ system itself is normal.<sup>164</sup> Red cells of patients with PNH exhibit augmented binding of C3<sup>155</sup> and increased sensitivity to lysis by the C5b-9 membrane attack complex (MAC), as do PNH platelets, granulocytes, and some lymphocytes.

The biochemical lesion in PNH is deficiency of PIG-anchored proteins in the affected cell membranes. The deficiency of DAF (CD55) impairs inactivation of the C3b convertase complex,<sup>165</sup> while reduced levels of MIRL (CD59) impair inactivation of the MAC.<sup>166</sup> Sensitivity to C′ varies inversely with levels of membrane expression of these proteins.<sup>167</sup> Lymphoblastoid cell lines developed from patients with PNH express the PNH defect and are incapable of synthesizing PIG core substance,

although they produce messenger RNA for PIG-anchored proteins. The specific abnormality in PNH is deficiency of uridine diphosphate-glucose-*N*-acetyl: phosphatidylinositol- $\alpha$ -1, 6-*N*-acetylglucosaminyltransferase.<sup>168</sup> A kindred has been described in which there is a hereditary deficiency of MIRL (CD59). A single member of this kindred, born to cousins, is homozygous for MIRL deficiency and expresses a PNH phenotype.<sup>169</sup>

The nature of the thrombotic tendency in PNH is incompletely understood.<sup>155</sup> Paroxysmal nocturnal hemoglobinuria platelets are partially activated by sublethal C'-mediated damage, and this activation may predispose to thrombosis.<sup>170</sup> However, because the clotting in PNH is principally venous,<sup>155,171</sup> the absent urokinase receptor may be important in decreasing the fibrinolytic system in resorbing venous clots.<sup>172</sup> Thrombocytopenia in PNH is attributable more to defective synthesis than excessive consumption in clotting.<sup>173</sup>

### Diagnosis

Paroxysmal nocturnal hemoglobinuria may be diagnosed by demonstration of chronic intravascular hemolysis attributable to C'-sensitive cells (deficient in PIG-anchored proteins) in the blood. Paroxysmal nocturnal hemoglobinuria should be considered in cases of hemolytic anemia with a negative DAT, aplastic anemia, and poorly understood pancytopenia or iron deficiency; hemolytic transfusion reactions in the face of a negative immunologic evaluation should also raise consideration of PNH. Atypical thromboses, leukopenia, and thrombocytopenia are subtle signs of PNH.<sup>174,175</sup>

### Laboratory Findings

Most patients with PNH have a cell volume of less than 30%, and many exhibit hypochromic, microcytic indices because of iron deficiency.<sup>156</sup> Reticulocytosis may be blunted because of the attendant iron deficiency. Elevated levels of serum bilirubin and lactic dehydrogenase are usual; haptoglobin levels are always reduced or absent. Virtually all patients have chronic hemosiderinuria, and absence of this finding should call the diagnosis into question. Granulocytopenia (cell count less than 1500/ $\mu$ L) is common.<sup>156</sup> The majority of patients also have mild thrombocytopenia (cell count less than 150,000/ $\mu$ L), with some platelet counts less than 50,000/ $\mu$ L.<sup>156</sup> The marrow cellularity varies from aplastic to hypercellular with erythroid hyperplasia. Maturation of marrow precursors is usually normal, but iron stores are characteristically reduced or absent.

### Confirmatory Tests

Several serologic tests demonstrate the C' sensitivity of PNH red cells. The acidified serum (Ham) test and the sucrose hemolysis test have been the standard confirmatory tests required for specific diagnosis.<sup>155,156,176</sup> More recently a variety of monoclonal antibodies that recognize the PIG-anchored proteins deficient in PNH have become available.

### Serologic Tests

In the acidified serum test, PNH red cells, but not normal cells, are lysed by normal human serum acidified to pH 6.5. With

appropriate controls this test is specific for PNH, but it is more time-consuming and technically difficult than the sucrose hemolysis test.<sup>176</sup> It rarely gives false-positive results in cases of hereditary erythrocyte multinuclearity with a positive acidified serum (HEMPAS) test or when intense spherocytosis is present.<sup>177</sup> In the sucrose hemolysis test, PNH red cells are lysed by C' components nonspecifically deposited on surfaces in solutions of low ionic strength. This test produces greater degrees of hemolysis than the acidified serum test, and values of 5% hemolysis or less are considered nonspecific and nondiagnostic.<sup>176</sup> These tests are complementary and should be performed in parallel if there is a high suspicion of PNH. Although the sucrose hemolysis test is more sensitive, it may fail to detect PNH in some patients whose cells do not exhibit increased sensitivity to C5-C9 MAC and whose acidified serum tests give positive results.<sup>176</sup>

### Immunophenotyping

Monoclonal antibodies have been developed to DAF (CD55) and to several other determinants on PIG-anchored proteins. These include MIRL (CD59), Fc $\gamma$ RIII (CD16), and CD48.<sup>178,179</sup> PIG-anchored proteins are expressed on a portion of PNH B cells, and a panel of monoclonal antibodies that detect CD55, CD59, and CD48 can be used to test for PNH.<sup>179</sup> Unlike traditional serologic tests, immunophenotyping may be applied to patients with PNH who develop aplastic anemia or are otherwise so heavily transfused as to have a low percentage of native PNH red cells. A rapid microtyping card test based on antibodies to CD55 and CD59 has been developed.<sup>180</sup> An improved method for the detection of PNH cells uses the selective binding of the protein aerolysin to GPI anchors; this is an important adjunct because no single monoclonal antibody can be used with confidence to diagnose PNH.<sup>181,182</sup>

### Pharmacologic and Immunotherapy

Treatment of PNH is determined by categorization of the disorder as a hypoplastic or classical form of PNH. Patients in the marrow failure group are managed as hypoplastic or aplastic anemia patients, depending upon the degree of aplasia. Hypoplastic variants require attention to correcting the cytopenias, while aplastic variants are treated with allogeneic stem cell transplants and/or immunosuppressive therapy. Patients with the classical form of PNH previously required allogeneic stem cell transplantation for attempted cure of the disorder. Eculizumab, a humanized monoclonal antibody against C5 that inhibits terminal complement activation,<sup>183</sup> is newly available for treatment of PNH. It is highly effective in reducing hemolysis and transfusional needs, in reducing the incidence of thrombosis, and in improving the quality of life in patients with PNH. A reduction in the levels of free hemoglobin reduces the nitric oxide consumption in PNH and helps eliminate the common symptoms of esophageal spasm and erectile dysfunction that complicate the disorder. Patients require vaccination against *Neisseria meningitidis* because of the increased risk of meningococcal infection with blockade of the terminal complement components.<sup>184,185</sup>

Hematinics

Chronic hemolysis in PNH often produces iron and folic acid deficiency, which may worsen anemia. Patients with PNH should receive supplemental folic acid. Iron therapy carries some risk of exacerbating hemolysis because of production of a cohort of C'-sensitive red cells.<sup>186</sup> Administration of oral iron is usually not accompanied by severe acute hemolysis and may be sufficient to offset urinary losses. However, when iron loss is more severe, some patients lose as much as 20mg/day,<sup>186</sup> and parenteral iron administration can become necessary. A severe acute hemolytic response to iron therapy often can be prevented by corticosteroid administration<sup>186</sup> (Table 22-5).

Androgens

A minority of patients with PNH respond to treatment with androgens.<sup>187</sup> These drugs may function by decreasing hemolysis or by increasing red cell production. Methyltestosterone, parenteral preparations, and synthetic steroids (including danazol) all have been effective in some patients but they have serious side effects, including cholestatic jaundice and peliosis hepatis, and predispose to progressive hepatic venous thrombosis. Because the response to androgens cannot be predicted, a trial of fluoxymesterone (10 to 30mg/day) or oxymetholone (10 to 50mg/day) is worthwhile, but these drugs should be discontinued if no response has occurred in 6 to 8 weeks.<sup>187</sup>

Corticosteroids

Steroids suppress hemolysis in the majority of patients with PNH. The onset of action generally occurs within hours, and 57% to 67% of patients respond to therapy with moderate-dose alternate-day oral prednisone.<sup>187,188</sup> The mechanism of steroid action is uncertain. Steroids may reduce C' activation by the alternate pathway, but red cells of patients with PNH who are taking prednisone are no less sensitive to C'-mediated lysis in standard in-vitro tests. Generally,

Table 22-5. Management of Paroxysmal Nocturnal Hemoglobinuria

| Agent or Therapy | Considerations                                                                                                                                                                                               |
|------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Hematinics       | Folic acid supplementation: <i>cautious</i> iron replacement                                                                                                                                                 |
| Androgens        | Minority of patients have dramatic reduction in anemia                                                                                                                                                       |
| Corticosteroids  | One-half to two-thirds of patients benefit from alternate-day therapy; heparin is used for acute episodes but may sometimes increase hemolysis; thrombolytic agents may be useful in hepatic vein thrombosis |
| Immunotherapy    | Eculizumab decreases hemolysis, lessens nitric oxide-related symptoms, and reduces incidence of thrombosis                                                                                                   |
| Transfusions     | For refractory symptomatic anemia or to abort or avert an acute hemolytic crisis; washed or frozen deglycerolized cells should be used                                                                       |

prednisone doses of 20 to 60mg/day are required to suppress hemolysis. Corticosteroids may be given daily for a limited time to achieve an immediate effect. However, their side effects, particularly predisposition to infection in patients already compromised by granulocytopenia, can be toxic. Corticosteroids should be prescribed for prolonged periods only on an alternate-day schedule and should be discontinued if they are ineffective after a trial of 2 to 4 weeks.

Splenectomy

There are potential hazards of surgical procedures in patients with PNH but, with careful attention to anesthetic and surgical technique, surgery can be accomplished with safety.<sup>189</sup> Splenectomy is usually not useful in PNH. In some patients, however, splenectomy reduces the anemia associated with PNH. This procedure is worth considering in patients with myelofibrosis-associated PNH.

Erythropoietin and Cytokines

Biologic agents have been used in a few patients with PNH. Although erythropoietin levels are usually elevated in patients with PNH, chronic anemia in patients with PNH sometimes responds to erythropoietin at doses between 50 and 500 U/kg given three times weekly.<sup>190</sup> Filgrastim (granulocyte colony-stimulating factor) administration led to recovery or protection from infection of two patients with PNH subject to repeated bacterial infection, and granulocyte colony-stimulating factor administration produced increased expression of PIG-anchored FcγRIII expression on their leukocytes.<sup>191</sup> The use of these agents in the routine care of patients with PNH remains to be defined but offers the hope of decreased demand for transfusion therapy and better outcomes to infectious episodes.

Marrow Transplantation

Clinical cures of PNH by allogeneic or syngeneic marrow transplantation have been increasingly reported, and expression of normal levels of PIG-anchored proteins on circulating blood cells of a transplant recipient with PNH has been directly demonstrated.<sup>192</sup> Allogeneic or syngeneic transplantation seems to be very effective for patients with PNH in whom aplastic anemia has supervened. Allogeneic transplants can restore normal marrow function in about 50% of patients with PNH.<sup>193</sup> The recent demonstration of a population of normal (DAF+ and CD59+) primitive (CD34+ and CD38+) cells in the marrow of patients with PNH suggests the possibility of selecting normal stem cells for autologous transplantation.

Anticoagulants and Thrombolytic Agents

Patients with PNH have a marked tendency for repeated venous thromboses,<sup>171</sup> requiring aggressive use of anticoagulants. Warfarin should be given as soon as such a complication is recognized. Heparin therapy is required for hepatic venous thrombosis and has been used without incident in many patients with PNH, although heparin does precipitate hemolytic episodes in some individuals.<sup>187</sup> Low-molecular-weight heparin may be more useful

for both the inhibition of hemolysis and the prevention of thrombosis in PNH.<sup>194</sup> Plasminolytic therapy has been used successfully to treat hepatic venous thrombosis<sup>195</sup>; its efficacy is related to the acuteness or sub-acuteness of the thrombosis and when the lysis is attempted. Previous recommendations of restricting its use to within 2 or 3 days after the thrombotic event have now been extended to as long as 2 to 3 weeks with some success.<sup>196</sup>

## Transfusion

### Indications

Red cell transfusions are sometimes required for relief of symptoms in PNH when hemolysis produces anemia that cannot be corrected by the therapeutic maneuvers discussed above. Additionally, hypertransfusion to a cell volume of 35% to 40% can abort acute hemolytic crises refractory to corticosteroids.<sup>187</sup>

## Choice of Blood Components

### Red Cells

Transfusion of whole blood to patients with PNH can initiate hemolysis and worsen anemia. Infused plasma can increase C' levels, whereas whole blood and red cells contain white cell fragments that can be immunogenic and result in C' activation.<sup>197</sup> Therefore, washed cells have been recommended and many transfusion services follow this practice. Sirchia and Zanella<sup>198</sup> have presented evidence that white cell fragments are the element most likely to initiate hemolysis during transfusion of patients with PNH, and they advocate the use of blood components that have undergone leukocyte reduction by filtration.

It seems clear that many patients with PNH tolerate infusion of whole blood or red cells without ill effects, but the proportion of patients with PNH who may experience increased hemolysis is not clear. The use of blood components that have been leukocyte reduced by filtration seems to be safer, and the use of washed or frozen deglycerolized cells avoids the problem altogether.

### Platelet Concentrates

Thrombocytopenia is common in patients with PNH and can be severe (platelet count less than 20,000/ $\mu$ L) enough to require platelet transfusion for surgery or for bleeding after trauma. Platelet preparations should be leukocyte reduced by filtration. The possibility of a hemolytic episode should be anticipated but can be treated with corticosteroids or hypertransfusion. Patients with PNH who receive many transfusions of non-leukocyte-reduced RBCs are at risk of becoming alloimmunized and may become refractory to platelet transfusion, in which case HLA-matched or crossmatched platelets may be required.

## Special Situations

### Pregnancy

Many successful pregnancies have occurred in women with PNH. Two-thirds have a successful outcome, but maternal death occurs in approximately 10% of PNH-affected pregnancies. Mothers often experience accelerated hemolysis and venous thrombosis.<sup>199</sup> Increased red cell transfusion is usually necessary, and

many authors advocate prophylaxis against venous thrombosis in patients who are bedridden and possibly also in the peripartum period.<sup>200</sup> Because of multiple maternal red cell transfusions, hemolytic disease of the fetus and newborn is frequent.<sup>201</sup>

### Extensive Elective Surgery

When extensive surgery is planned for a patient with PNH, the possibility of severe hemolysis should be anticipated. An exchange transfusion can be performed before the operation. The precaution prevents hemolysis and provides the surgeon and anesthetist with a more stable patient.<sup>189</sup>

### Disseminated Intravascular Coagulation

Disseminated intravascular coagulation seems to be a rare direct complication of PNH.<sup>202</sup> When asymptomatic, it has been monitored carefully<sup>202</sup> or treated with blood component support. Additional special measures have not been required on account of PNH, and cryoprecipitate and platelet concentrates have been tolerated without accelerated hemolysis being noted.

### Myelofibrosis

As many as 50% of patients with primary myelofibrosis exhibit red cell membrane defects of PNH and some suffer from thrombotic complications.<sup>203</sup> These patients have marrow failure contributing to their anemia, in addition to hemolysis, and sometimes have substantial transfusion requirements. Contrary to the usual finding of iron deficiency, such patients can develop transfusion-related hemosiderosis.

### Acute Leukemia

Paroxysmal nocturnal hemoglobinuria evolves into acute myelocytic leukemia (AML) in 2% to 4% of patients.<sup>204</sup> Leukemia apparently develops in the PNH clone, and there is cytogenetic evidence of clonal evolution.<sup>205</sup> Complete remissions can be induced with antileukemic therapy, and in these patients there has been no evidence of PNH during remission.<sup>206</sup> Reports of patients with PNH who develop AML do not indicate increased complications referable to the PNH defect, despite the need for intensive transfusion support. In fact, in one patient who did not undergo induction chemotherapy for AML, complications of PNH decreased during the course of the leukemia.<sup>207</sup>

### Aplastic Anemia

Paroxysmal nocturnal hemoglobinuria may evolve from or into aplastic anemia.<sup>208</sup> Development of this complication is compatible with prolonged survival with treatment similar to that prescribed for uncomplicated cases of PNH.<sup>209</sup> Aplastic anemia associated with PNH may be successfully treated by marrow transplantation.

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## Summary

Autoimmune hemolytic anemia is divided into warm and cold autoantibody types and may be idiopathic (primary) or associated



with another disease (secondary). It also may be drug induced. The DAT is an important laboratory marker but may produce negative results in patients with anemia and positive results in patients without anemia. Transfusion frequently requires autoadsorption studies. Glucocorticoids and splenectomy are primary treatments, although immunosuppressive drugs and other treatments are also used. The cold autoantibody type of AIHA is either acute or chronic cold hemagglutinin disease or PCH. Drug-induced hemolytic anemia involves drug adsorption, a drug-dependent antibody, or autoantibody induction mechanisms.

Paroxysmal nocturnal hemoglobinuria is an uncommonly diagnosed hemolytic anemia that arises from a somatic mutation of pluripotent hematopoietic stem cells. Hemolysis results from increased sensitivity to C' produced by deficiency of the PIG-anchored proteins, including DAF and MIRL, in the red cell membrane. The acidified serum test, the sucrose hemolysis test, and immunophenotyping of blood cells are used for diagnosis. Various therapies including immunotherapy with eculizumab and marrow transplantation have been successfully used in PNH. The disorder is protean in its manifestations with hemolysis, thrombosis, infection, smooth muscle dystonias, aplastic anemia, and leukemia as part of its natural history.

## Disclaimer

The author has disclosed no conflicts of interest.

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### General Tests to Investigate Thrombocytopenia

Thrombocytopenia can be caused by platelet underproduction, sequestration, hemodilution, or destruction. This chapter explores the problem of immune platelet destruction. Immune thrombocytopenia is characterized by a shortened platelet life span caused by platelet-antibody interactions. Several indirect clues suggest a shortened life span, such as a rapid decrease in platelet count or severe thrombocytopenia in a patient with normal megakaryocyte numbers. Radioactive markers sometimes are needed to establish directly the existence of a shortened platelet survival time. An additional role for impaired thrombopoiesis is recognized increasingly in the pathogenesis of autoimmune thrombocytopenia.

### Complete Blood Cell Count and Blood Film

Platelets usually are quantitated during a complete blood cell count with a particle counter. A normal platelet count usually is 150,000 to 400,000/ $\mu$ L, although the reference range may be lower in Mediterranean populations (125,000 to 300,000/ $\mu$ L) that have larger-sized platelets. The platelet count usually remains fairly stable throughout a normal human life span.<sup>1</sup> An exception occurs during pregnancy, when the platelet count decreases somewhat, perhaps the result of increased plasma volume (hemodilution). An elevated platelet count also is normal 10 to 14 days after a major surgical procedure (postoperative thrombocytosis, 250,000 to 1,000,000/ $\mu$ L) before return to preoperative baseline by 3 weeks after the operation.<sup>2,3</sup> Thus, a platelet count of only 190,000/ $\mu$ L 10 days after an operation on a patient with dyspnea who had received postoperative heparin prophylaxis could represent pulmonary embolism complicating heparin-induced thrombocytopenia (HIT).

A useful general rule is that isolated thrombocytopenia usually is caused by increased platelet destruction, whereas bicytopenia or pancytopenia usually is attributable to marrow dysfunction,

hypersplenism, or hemodilution. Isolated, severe thrombocytopenia (platelet count less than 20,000/ $\mu$ L) often indicates platelet destruction by autoantibodies, alloantibodies, or drug-dependent immunoglobulin G (IgG) antibodies. Such severe thrombocytopenia, however, occasionally occurs in patients with HIT<sup>4</sup> or septicemia, although platelet count nadirs are typically more than 20,000/ $\mu$ L in these two disorders characterized by in-vivo platelet activation caused by heparin-dependent IgG antibodies or thrombin, respectively. Examining the blood film is important to exclude pseudothrombocytopenia (spurious thrombocytopenia resulting from antibodies that cause ex-vivo platelet agglutination) and to suggest various nonimmune causes of thrombocytopenia, such as toxic leukocytes indicating infection or fragmented red cells suggesting microangiopathic hemolysis. In contrast, immune thrombocytopenia usually is characterized by reduction in platelet numbers with otherwise unremarkable morphologic features, unless the thrombocytopenia is secondary to a disorder such as lymphoma.

### Platelet Size, Platelet RNA, and Plasma Glycocalicin

A particle counter also is used to determine average platelet size, or mean platelet volume (MPV), which usually ranges from 7.0 to 10.5 fL. Disorders of increased platelet destruction usually are characterized by large platelets, and MPV ranges from 10 to 15 fL. Normal-sized or small platelets are common in disorders of underproduction or sequestration of platelets.

Young platelets contain residual amounts of RNA, which can be detected by means of flow cytometric analysis of platelets labeled with either thiazole orange or auramine-O. However, such quantitation of reticulated platelets has not gained the acceptance that red cell reticulocyte assays have. One problem is that thiazole orange also labels platelet-dense granules nonspecifically.

Levels of glycocalicin [a soluble proteolytic product of platelet glycoprotein Ib (GPIb)] are increased in patients with increased platelet destruction. Because of factors such as platelet count, renal function, disease-related proteolysis, and technical considerations, this assay is used predominantly for research.<sup>5</sup>

## Marrow Examination

Disorders of increased platelet destruction are characterized by normal or increased numbers of megakaryocytes in the marrow. Sometimes examination of the marrow yields enough information to determine the cause of the thrombocytopenia, such as myelodysplasia or megakaryoblastic anemia.

## Measurement of Platelet Life Span

Measurement of the life span of platelets is the definitive test for classifying the cause of thrombocytopenia. Indium-111 is the radiolabel of choice because of its higher labeling efficiency and efficient range of  $\gamma$  emissions. Indium-111 is not released from platelets by platelet autoantibodies. Three patterns of platelet survival can be observed, as follows: 1) normal platelet recovery (60% to 75%) and a normal survival time (7 to 10 days) characterize thrombocytopenia caused by underproduction; 2) a markedly reduced platelet life span (sometimes only hours) is found in patients with thrombocytopenia caused by increased platelet destruction; and 3) reduced platelet recovery (as low as 10% to 30%) with a normal or near-normal platelet survival time confirms the diagnosis of thrombocytopenia caused by increased platelet sequestration (hypersplenism). Platelet life span studies are not often performed, however, because physicians usually infer the mechanism of the thrombocytopenia from the clinical situation.

## Platelet-Antibody Assays

There are two broad categories of platelet-antibody assays—classical platelet-associated IgG assays and assays that identify the protein target of the antibody (protein-specific assays).

### Classical Platelet-Associated Immunoglobulin G Assays

Measurement of platelet-associated IgG (PAIgG) has been widely available for 25 years. Unfortunately, these assays have limited diagnostic usefulness. A positive assay result does not differentiate immune from nonimmune thrombocytopenia.<sup>6</sup> These assays can detect either surface-associated immunoglobulin or complement by means of direct binding of a labeled anti-immunoglobulin (and/or anticomplement) probe, or total PAIgG measured after platelet lysis.

#### Direct Binding Assays for Platelet Surface Immunoglobulin G

In direct binding assays, binding of a labeled anti-immunoglobulin probe to the platelet surface is quantitated. The anti-immunoglobulin probe is labeled with radioisotope, fluorescent marker, or an enzyme. The labeled probe (eg, anti-IgG, anti-IgM, or anticomplement) is incubated with washed test platelets, unbound probe is washed away, and the amount bound to the platelets is measured.<sup>6</sup> Although these assays are simple, a disadvantage is that platelet membranes nonspecifically adsorb proteins, including the labeled probe. However, there is a more fundamental problem: even monoclonal anti-immunoglobulin

probes, despite their low nonspecific binding to platelet membranes, cannot differentiate immune from nonimmune thrombocytopenic disorders.<sup>6</sup> This type of assay can be diagnostically useful, however, in special situations, such as detecting a drug-dependent increase in platelet surface-bound IgG in the presence of patient serum.<sup>7</sup>

### Total Platelet-Associated Immunoglobulin G

The total amount of IgG, from both the interior and the surface of platelets, can be measured as total PAIgG.<sup>6</sup> The test platelets are washed and counted, and the platelet membranes are lysed. The total amount of PAIgG in soluble form can be measured with any technique used to measure fluid-phase IgG, such as nephelometry or immunoradiometric assay. However, the limited diagnostic value of this assay is not surprising, given that less than 1% of PAIgG is bound to the platelet surface, indicating that the majority of IgG measured is nonspecific and stored internally.

### Protein-Specific Platelet-Antibody Assays

The diagnostic usefulness of platelet-antibody assays has increased dramatically with the introduction of various protein-specific assays that help identify the platelet protein target of antibodies with either monoclonal antibodies or electrophoretic techniques.

### Monoclonal Antibody Assays

Various monoclonal antibody-based assays can be used to detect platelet antibodies. Perhaps most widely used is the monoclonal antibody immobilization of platelet antigen (MAIPA) assay. An improved modified MAIPA assay<sup>8</sup> is shown in Fig 23-1(A). A technically simpler assay is the antigen capture enzyme immunoassay, in which platelet glycoprotein monoclonal antibodies interact with detergent-solubilized platelet samples [Fig 23-1(B)], rather than intact platelets.<sup>7,9</sup>

Simplicity and relatively high diagnostic specificity are advantages of these assays, especially the antigen capture assay. In addition to investigation of autoimmune thrombocytopenia, these assays can be adapted for study of alloimmune disorders with a panel of platelet glycoproteins of known alloimmune phenotype, or for study of drug-induced thrombocytopenia through demonstration of drug-dependent binding of antibody to platelet glycoproteins.<sup>7</sup> A disadvantage, however, is that they detect antibodies that bind to platelet proteins captured with the specific monoclonal antibody, so a large number of different monoclonal reagents are needed. These assays also can give false-negative results if the patient antibody binds to the same epitope recognized by the monoclonal antibody. Some human sera contain antibodies that recognize murine IgG, which is why the modified MAIPA and antigen capture assays are preferred to the original MAIPA.<sup>8</sup>

### Immunoprecipitation

Immunoprecipitation is performed by incubating patient serum or plasma with platelets labeled with iodine-125 or tagged with nonradioactive biotin.<sup>10</sup> The proteins are then solubilized by the addition of detergent, and the antibody-protein complex is



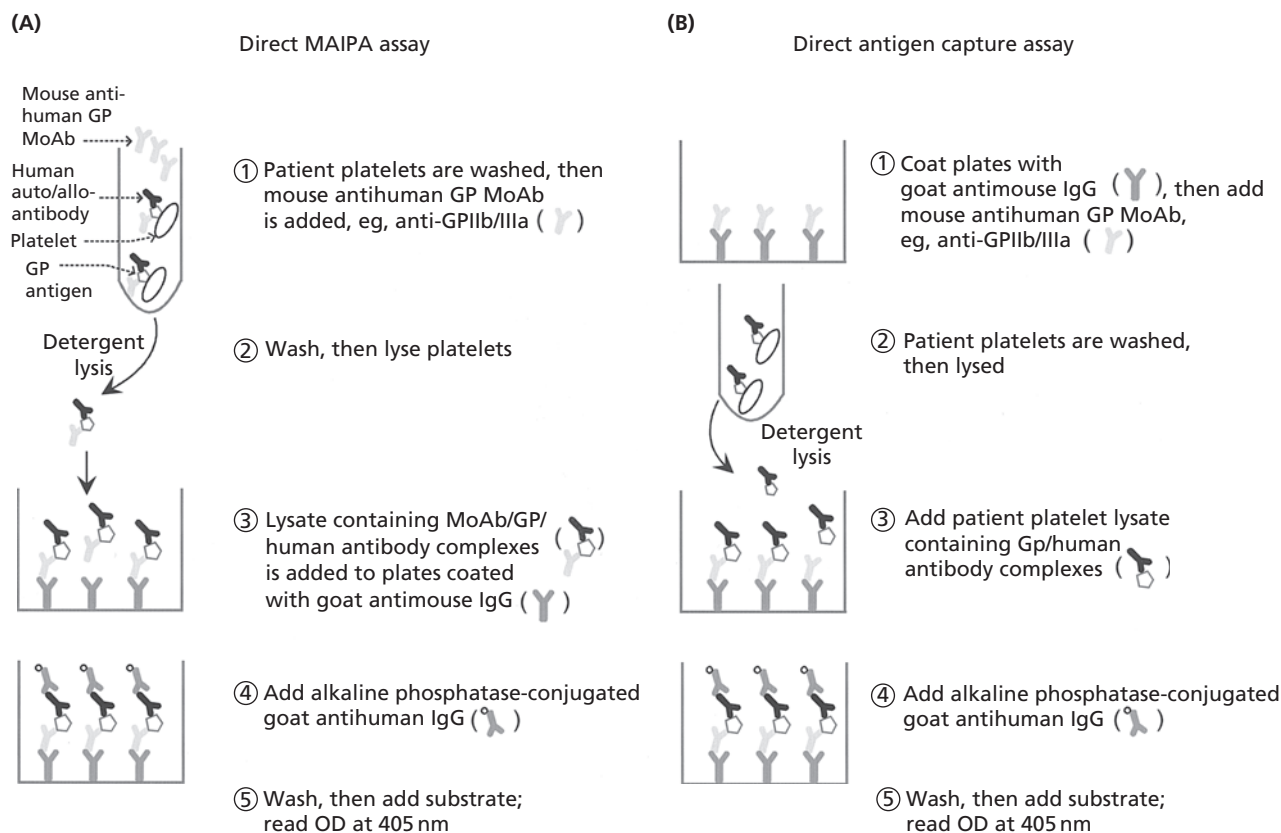


**Figure 23-1.** Modified monoclonal antibody immobilization of platelet antigen (MAIPA) assay and antigen capture assay. “Direct” versions of both assays are shown, that is, the patient’s platelets are tested for the presence of platelet glycoprotein (GP)-bound autoantibodies. (In indirect assays, the patient’s serum or plasma is added to target platelets to detect the presence of serum or plasma platelet glycoprotein antibodies.) (A) In the MAIPA assay, the antihuman GP monoclonal antibodies (MoAbs) are added to washed intact patient platelets

before detergent lysis.<sup>8</sup> (B) In the antigen capture assay, the patient’s platelets are washed, then lysed, and only later is GP MoAb added.<sup>9</sup> However, both assays resemble each other in the final stages (steps 4 and 5). A relative advantage of the antigen capture assay is that the platelet lysate can be stored and the assay performed later using any MoAb of choice, making this a technically simpler and more versatile assay. OD = optical density.

precipitated by addition of an anti-immunoglobulin bound to a solid phase (eg, immobilized staphylococcal protein A). The labeled protein-antibody complexes are washed, and the platelet proteins are separated by denaturing gel electrophoresis and detected by autoradiography or use of enzyme-conjugated streptavidin. The target antigen is identified according to its electrophoretic mobility. Either the patient’s platelets are used (direct immunoprecipitation), or patient serum or plasma is mixed with target platelets (indirect immunoprecipitation).

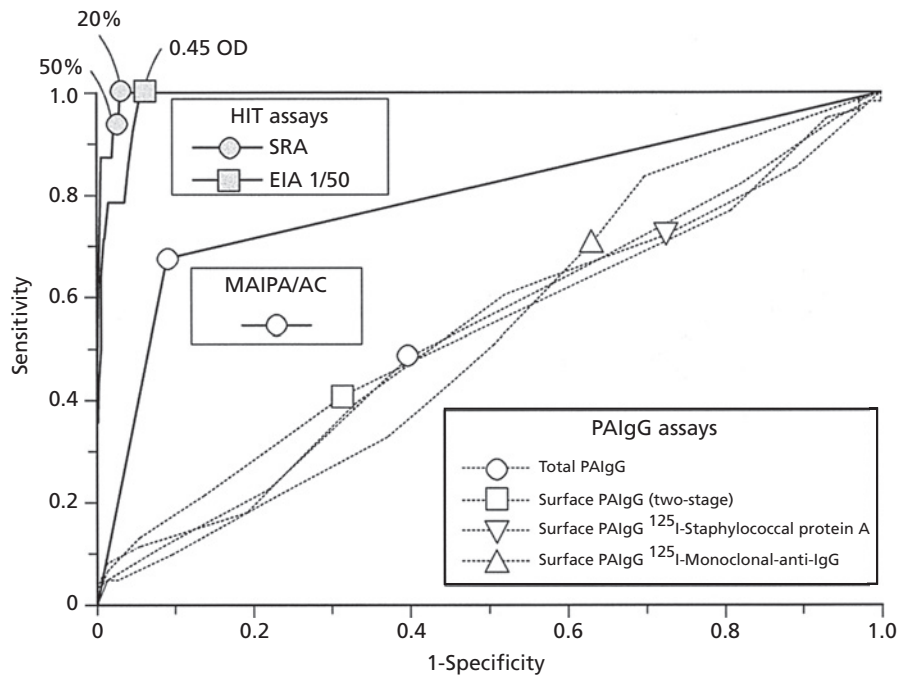
Immunoprecipitation offers advantages over immunoblotting because the antibody reacts with native, rather than denatured, platelet proteins. In particular, this technique has allowed detection of clinically significant antibodies against previously unrecognized platelet proteins, such as anti-HPA-15 (anti-Gov) on CD109, a 175-kD glycosylphosphatidylinositol (GPI)-anchored protein.<sup>11</sup> The main disadvantage of immunoprecipitation is

technical difficulty. In addition, not all platelet proteins are optimally labeled (eg, GPVI).

### Immunoblotting (Western Blotting)

In immunoblotting, patient serum is allowed to interact with platelet proteins that have been electrophoretically separated and then immobilized onto a solid phase (nitrocellulose paper). The test serum is added and binding of patient antibody to specific protein bands is detected with labeled anti-immunoglobulin. Although immunoblotting offers the advantage of simplicity and the opportunity to store the immobilized proteins for long periods, a major disadvantage is that protein antigens are denatured in this method. This can destroy some platelet antigens (eg, HPA-5 and -15) and may modify or expose epitopes to which normal sera can react (eg, vinculin, talin), making interpretation difficult.<sup>11,12</sup>

**Figure 23-2.** Operating characteristics of various platelet-antibody assays. The operating characteristics (sensitivity-specificity trade-offs at various diagnostic cutoffs between positive and negative results) show that assays for heparin-induced thrombocytopenia (HIT) are diagnostically useful (high sensitivity and specificity).<sup>2,13,14</sup> The data for the HIT assays show two diagnostic cutoffs (20% and 50% serotonin release) for the serotonin release assay (SRA), and one diagnostic cutoff (0.45 optical density unit) for the platelet factor 4/heparin enzyme immunoassay (EIA, performed with serum at 1/50 dilution). In contrast, the modified monoclonal antibody immobilization of platelet antigen (MAIPA) assay and antigen capture (AC) tests have moderate usefulness for diagnosis of autoimmune thrombocytopenia (high specificity but low to moderate sensitivity).<sup>9</sup> Conventional assays for platelet-associated immunoglobulin G (PAIgG) have minimal diagnostic value;<sup>6</sup> that is, sensitivity is similar to 1 – specificity. Open symbols = diagnostic cutoff actually used for each assay. The data used for the MAIPA, AC, and PAIgG assays are from published sources.<sup>6,9</sup> Used with permission from Warkentin.<sup>13</sup>



### Tests for Heparin-Induced Thrombocytopenia

Special tests are required to detect the antibodies that cause HIT. These assays are classified as platelet activation or platelet factor 4 (PF4)-dependent antigen assays.<sup>13,14</sup>

#### Platelet Activation Assays

Heparin-induced thrombocytopenia antibodies have potent heparin-dependent, platelet-activating properties. Aggregation of platelets (prepared as citrate-anticoagulated platelet-rich plasma) by patient plasma detected with conventional platelet aggregometry once was widely used for HIT; however, the sensitivity of this assay is relatively low for detecting HIT antibodies. In contrast, assays performed with washed platelets that have been resuspended in calcium-containing buffer, such as the platelet serotonin release assay (SRA) or heparin-induced platelet activation assay, are both sensitive and specific for detecting clinically significant HIT antibodies.<sup>13,14</sup> However, these assays are not widely available because they are technically demanding, require careful platelet donor selection, and require a panel of strong and weak positive serum controls. Heparin-induced thrombocytopenia antibodies have a characteristic activation profile: platelet activation at therapeutic, but not high, heparin concentrations and inhibition of activation by Fc receptor-blocking monoclonal antibodies.

#### Platelet Factor 4/Heparin Enzyme Immunoassay

In 1992, Amiral et al<sup>15</sup> identified PF4/heparin complexes as the antigen of HIT. Both commercial and in-house antigen assays based on enzyme immunoassay (EIA) methods have since become available.

An intriguing feature of the HIT antigen is that certain polyanions other than heparin render PF4 antigenic. This is the basis of the PF4-polyvinylsulfonate EIA for PF4/heparin antibodies.<sup>16</sup>

#### Diagnostic Usefulness of Platelet-Antibody Assays

Figure 23-2 compares the operating characteristics (sensitivity-specificity trade-offs) for three types of platelet-antibody assays. Classical PAIgG assays provide limited diagnostic information,<sup>6</sup> whereas the newer protein-specific platelet-antibody assays have moderate sensitivity and relatively high specificity for immune thrombocytopenia.<sup>9</sup> Both the antigen and washed-platelet activation assays are useful for diagnosis of HIT.<sup>13,14</sup>

### Platelet Genotyping

Serologic assays are generally reliable for detecting platelet alloantibodies. However, a disadvantage of antibody-based analysis is that there may not be sufficient platelets available from a patient with severe thrombocytopenia [eg, posttransfusion purpura (PTP)] to allow determination of the reciprocal platelet alloantigen phenotype.<sup>17</sup> Molecular techniques provide a reliable alternative to serologic phenotyping. Genomic DNA is used to determine the corresponding platelet alloantigen *genotype* and is readily available from a number of sources. Molecular techniques are useful in the evaluation of suspected neonatal alloimmune thrombocytopenia (NAIT). Analysis of fetal cells (obtained by amniocentesis, chorionic villus sampling, or sampling of fetal blood) can determine whether a fetus is at risk for

this complication. Small amounts of tissue can be studied (eg, 5 to 10 mL of amniotic fluid), because the technique of polymerase chain reaction (PCR) greatly amplifies the DNA. The possibility of significant maternal DNA contamination of a fetal sample can be assessed with the forensic technique of variable-number tandem repeat analysis of the sample.<sup>18</sup>

### Polymerase Chain Reaction and Restriction Fragment Length Polymorphism

For all platelet alloantigen polymorphisms but one (HPA-4), the single-base substitution responsible for the change in the expressed amino acid is associated with a restriction endonuclease recognition site.<sup>17</sup> Accordingly, restriction fragment length polymorphism (RFLP) analysis was the first genotyping assay developed to identify platelet alloantigens. In the PCR-RFLP method, a section of DNA that encompasses the polymorphism is amplified using a pair of sense/antisense primers. When the amplified fragment is subjected to restriction enzyme digestion and separated by means of agarose gel electrophoresis, the position and number of fragments indicate the platelet antigen genotype. One limitation is the possibility that another polymorphism within the amplified fragment could confound the genotyping by interfering with the restriction enzyme, as has been shown for the *Msp* I site for HPA-1a/1b.<sup>19</sup>

### Allele-Specific Polymerase Chain Reaction

Because PCR-RFLP is a comparatively labor-intensive technique, an alternative is widely used. In allele- or sequence-specific PCR (SSP-PCR), one of the primers is specific for the particular allele to be amplified.<sup>20</sup> When the allelic polymorphism is positioned at the 3' end of the oligonucleotide primer, efficient amplification occurs only when the primer is 100% complementary to the genomic sequence. *Taq* polymerase is used for the PCR because this enzyme does not repair single-base mismatches at the 3' end of the primers that occur when the mismatch is present. Thus, each allele-specific primer with a common universal primer will amplify the DNA only if the allele is present. The advantage of this method is that the PCR product is visualized directly in agarose gels to determine the genotype. Appropriate controls must be included in the assay, including additional primers to amplify a ubiquitous gene (eg, human growth hormone) to ensure that all assay constituents are working properly.

### Real-Time Polymerase Chain Reaction

A modification of SSP-PCR is real-time PCR. Real-time PCR has improved both the speed and accuracy of genotyping.<sup>21</sup> The use of specifically designed hybridization probes tagged with fluorescent dyes for the PCR allow direct determination of the platelet genotype without gel electrophoresis or restriction enzymes, and in a single reaction. One hybridization probe, the donor, is tagged with a dye (fluorescein) and emits light at a specific wavelength when excited by a light source. The other probe, the acceptor, is tagged with a different dye. It straddles the single nucleotide polymorphism of interest and is 100% homologous to one of the platelet alleles. The acceptor probe usually is one nucleotide away from the donor probe in a head-to-tail arrangement; that is, the

dyes are juxtaposed. The energy emitted by the dye of the donor probe is transferred to the adjacent dye of the acceptor probe, which emits light at a different wavelength. The intensity of the second light emitted is proportional to the amount of double-stranded DNA present. Unbound donor probe in the mixture can be excited but cannot transfer energy to the acceptor probe. At the end of the PCR, the platelet genotype is determined by means of melting curve analysis. Because the acceptor probe straddles the polymorphism and is 100% homologous to one of the alleles, the melting curve has a different temperature midpoint ( $T_m$ ) depending on whether a mismatch is present. The acceptor probe can have as much as 5°C to 8°C difference in  $T_m$  between the two platelet alleles. In all, three melting curves are seen, one for each polymorphism, and a composite melting curve when the heterozygous situation is present.<sup>21</sup> The advantage of fluorescence-based real-time PCR with melting curve analysis is that the PCR product is measured directly and no further manipulation is required. Any additional polymorphism in the region of the acceptor probe is detected in the melting curve analysis. Rapid thermal cycling and DNA extraction permits determination of a platelet genotype within 2 hours of specimen collection.

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## Pathophysiology of Immune Platelet Destruction

Immune thrombocytopenia results from antibody-mediated platelet destruction. The IgG-sensitized platelets are phagocytosed by monocytes and macrophages of the reticuloendothelial system. Reticuloendothelial cells are located throughout the body but are concentrated in the spleen, liver, lungs, and marrow. Pathogenic antibodies bind to platelets via their Fab termini, usually against specific autoantigen or alloantigen epitopes. Sometimes the target antigen is induced by a drug or drug metabolite. The result is a ternary complex that involves IgG, drug, and a specific region on a platelet glycoprotein. Heparin-induced thrombocytopenia is an exception to these generalizations: although HIT antibodies bind to PF4/heparin complexes via the Fab terminus, the Fc moiety of IgG interacts with platelet FcγIIa receptors, resulting in platelet activation.<sup>4</sup>

The rate of platelet destruction is determined by the quantity and subclass distribution of IgG on the platelet, the amount of complement, and the efficiency of reticuloendothelial clearance. The severity of thrombocytopenia reflects the balance between the rate of platelet destruction and the compensatory marrow thrombopoiesis.

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## Idiopathic (Immune) Thrombocytopenic Purpura

Immune thrombocytopenic purpura (ITP), also known as primary autoimmune thrombocytopenic purpura, is a common disorder characterized by increased platelet destruction and impaired platelet production. It is primarily a diagnosis of exclusion, defined as isolated thrombocytopenia with no other

clinically apparent cause such as human immunodeficiency virus (HIV) infection, systemic lupus erythematosus (SLE), drug-induced thrombocytopenia, alloimmune thrombocytopenia, lymphoproliferative disorders, myelodysplasia, hypogammaglobulinemia, splenomegaly, or familial thrombocytopenia.<sup>22</sup> Although platelet-antibody assays allow detection of platelet GP-reactive autoantibodies in up to 75% of patients with ITP, the false-negative rate (at least 25%) means that a negative test does not exclude the diagnosis of ITP.<sup>9,23</sup>

### Pathogenesis

Over 50 years ago, Harrington et al<sup>24</sup> showed that ITP plasma infused into healthy volunteers caused acute severe thrombocytopenia. The platelet-destroying plasma factor was later shown to be IgG, although in some patients, IgM and IgA antibodies may be pathogenic. The immune target of the autoantibodies usually is one of the two major platelet glycoprotein complexes, with GPIIb/IIIa implicated more often than GPIb/IX.<sup>25</sup> Other less common autoantigen targets include GPIa/IIa and, possibly, non-protein targets such as glycosphingolipids. The autoantibodies bind to platelets by way of their Fab terminus and cause premature platelet destruction via Fc receptor (Fc $\gamma$ R)-mediated phagocytosis by macrophages in the spleen and other reticuloendothelial tissues. Platelet autoantibodies can also cause binding of complement *in vitro*; however, *in-vivo* complement-mediated platelet lysis has not been proven. Another proposed mechanism of platelet destruction is direct platelet lysis by T cells.<sup>26</sup>

Platelet production is also impaired in ITP. Platelet turnover is lower than expected, as shown by radiolabeled autologous platelet studies.<sup>27</sup> Further, levels of thrombopoietin (TPO), the constitutively expressed, principal thrombopoiesis regulatory hormone, are lower in ITP compared with other thrombocytopenic disorders such as aplastic anemia.<sup>28</sup> An increased megakaryocyte mass may account for the relative TPO deficiency, because these cells bind free TPO; conversely, platelet underproduction may result from megakaryocyte injury caused by autoantibodies.<sup>29</sup>

A fundamental research problem in ITP is to understand the relation between pathogenic platelet-reactive autoantibodies and measured PAIgG. Doubt regarding the pathogenic significance of elevated PAIgG levels was raised by the observation that even nonimmunoglobulin proteins, such as albumin, are elevated on platelets of patients with ITP.<sup>30</sup> Results of subsequent investigations suggested that elevated PAIgG and albumin levels in ITP and other disorders with increased platelet turnover are caused by increased endocytosis of these plasma proteins into platelet  $\alpha$  granules, which occurs either in megakaryocytes or mature platelets.<sup>31,32</sup> Electron microscopic examination and immunogold labeling of IgG show increased amounts of IgG within  $\alpha$  granules, as well as the open canalicular system and platelet surface, of patients with ITP.<sup>33</sup> Newer assays that detect glycoprotein-specific antibodies are more informative.<sup>23</sup>

The cause of autoantibody formation in ITP is not known. Light-chain and immunoglobulin subclass restriction of

platelet-reactive antibodies suggests an oligoclonal origin of autoantibodies in chronic ITP.<sup>34</sup> Further, the autoepitopes involved may be fairly narrow in scope. Autoantibodies are produced by B lymphocytes, and the loss of T-cell tolerance to platelet proteins is a key feature.

### Clinical and Laboratory Features

Patients are arbitrarily considered to have chronic ITP when thrombocytopenia has lasted more than 6 months. Although chronic ITP can occur at any age and in both sexes, women between 30 and 50 years of age are affected most often (female to male ratio, approximately 3:1). This condition usually is asymptomatic, or the symptoms and signs are limited to bleeding. If clinical evaluation reveals weight loss, fever, lymphadenopathy, hepatomegaly, or splenomegaly, other diagnoses should be considered. Some physicians perform routine screening for thyroid disease,<sup>22</sup> because both hyper- and hypothyroidism are associated with ITP.

The onset of bleeding symptoms is typically insidious. Mucocutaneous bleeding is the hallmark of ITP and manifests as purpura (petechiae, ecchymosis), epistaxis, menorrhagia, oral mucosal bleeding, and gastrointestinal bleeding. Aspirin ingestion can cause bleeding in a patient who otherwise has no symptoms. Immune thrombocytopenic purpura is rarely life-threatening; for example, in a pooled analysis of ITP patients with persistent low platelet counts (less than 30,000/ $\mu$ L), the rate of fatal bleeding (usually from intracranial hemorrhage) was estimated at 0.02 to 0.04 case per patient-year, but was highest (approximately 0.13 case per patient-year) among patients over 60 years of age.<sup>35</sup> On the other hand, many patients even with severe thrombocytopenia have minimal or no bleeding, possibly because of the presence of small numbers of young, reticulated platelets with enhanced  $\alpha$ -granule release to platelet agonists.<sup>36</sup> Often, morbidity is attributable to the treatments and not the disease itself.<sup>37</sup>

The complete blood count usually reveals isolated thrombocytopenia, although microcytic anemia can indicate iron deficiency in patients with chronic bleeding. The MPV usually is increased, although some patients with severe ITP can have a normal or even low MPV. The marrow examination shows normal or increased megakaryocyte numbers, although diagnostic marrow examinations are generally needed only for elderly patients in whom myelodysplasia is common. The bleeding time usually is elevated only when the platelet count is less than 30,000/ $\mu$ L. An elevated bleeding time with a high platelet count is rarely caused by autoantibodies that interfere with platelet glycoprotein function. About 15% to 25% of patients with otherwise typical ITP have a positive result of an antinuclear antibody test, usually low titer. These patients generally do not have a different clinical outcome than do patients with negative antinuclear antibody results.<sup>38</sup> Although platelet autoantibodies are generally not needed to diagnose ITP, direct assays that detect glycoprotein-specific platelet autoantibodies on the platelet surface are more sensitive than indirect assays using patient serum or plasma (~75% vs 50% sensitivity, respectively).<sup>23</sup>

## Treatment Overview

The principles of treatment of adults with ITP are based on the following considerations:

1. Most patients with ITP have hyperfunctional platelets associated with an increased MPV. Thus, many patients do not need treatment despite moderate or even severe thrombocytopenia.
2. The goal of treatment is to achieve a safe platelet count, not necessarily a normal count. The presence of petechiae is helpful in assessing the need for treatment of a patient with minimal bleeding. Prophylactic platelet transfusions are rarely indicated in the management of ITP. Although a transient increase in platelet count usually can be achieved,<sup>39</sup> the benefits of a short-lived increase in a chronic disorder are doubtful. However, platelet transfusions are indicated in the care of patients with life-threatening bleeding (see later).
3. Most adults have chronic ITP; the rate of spontaneous remission is estimated to be only 5%.<sup>22</sup> Thus, the risks of treatment must be balanced against the risks of life-threatening bleeding. Most adults are not cured by a prolonged course of prednisone but often develop significant morbidity from this agent. Splenectomy offers a good chance for a long-term cure and avoids the long-term toxicity of medical therapy.
4. Treatment with corticosteroids and splenectomy fail in approximately 10% to 15% of patients. For these patients, numerous alternative treatments are available, including intravenous immune globulin (IVIG), intravenous Rh Immune Globulin (WinRho, Cangene, Winnipeg, Canada or Rhophylac, ZLB Bioplasma AG, Berne Switzerland), rituximab, danazol, vinca alkaloids, cyclophosphamide, azathioprine, and cyclosporine, among others. Recently, TPO agonists have also been shown to be effective in early clinical trials.
5. Drugs that interfere with platelet function—particularly aspirin, nonsteroidal antiinflammatory agents, and alcohol—should be avoided.

## First-Line Therapy

### Corticosteroids

Corticosteroids improve platelet survival in ITP by reducing both reticuloendothelial phagocytic activity and synthesis of pathogenic autoantibodies.

The standard initial dosage of prednisone is 1 mg/kg/day, usually given until the platelet count has increased to safe values. Regardless of the response, tapering usually is started on Day 14 of therapy, if not sooner. Two prospective studies<sup>40,41</sup> compared conventional and lower-dose prednisone therapy (1 mg/kg/day vs 0.25 mg/kg/day in one trial; 1.5 mg/kg/day vs 0.5 mg/kg/day in the other), and in neither study was there a significant difference in remission at 6-month follow-up evaluation. In the larger study,<sup>41</sup> however, the higher-dose regimen showed a trend to a higher rate of complete remission (46% vs 35%) as well as higher platelet counts at 14-day follow-up evaluation (77% vs 51% having a platelet count greater than 50,000/ $\mu$ L).

For patients with newly diagnosed ITP, very large doses of corticosteroids have been used with the hope of achieving durable

remissions. Initial treatment with high-dose pulse methylprednisolone (starting at 30 mg/kg/day intravenously) resulted in better short-term platelet count responses compared with conventional doses, but long-term responses were not improved.<sup>42,43</sup> In one study ( $n = 125$ ), 84% of patients with acute ITP treated with a single course of high-dose dexamethasone (40 mg/day for 4 days) achieved a platelet count response (to greater than 50,000/ $\mu$ L) and of those, 50% had a response that lasted from 2 to 5 years.<sup>44</sup> In another study ( $n = 37$ ), six courses of monthly high-dose dexamethasone resulted in an overall response rate of 83.8%, and a sustained response of 64.9% after 2 years.<sup>45</sup> However, repeated cycles of high-dose dexamethasone were often poorly tolerated.

In patients with chronic ITP, responses with high-dose dexamethasone are variable. In one study, all 10 treated patients achieved a response that lasted 6 months following six cycles of high-dose dexamethasone (40 mg/day for 4 days).<sup>46</sup> However, other subsequent studies were less encouraging. Using the same regimen, Stasi et al<sup>47</sup> reported that 13 of 32 patients (40.6%) had transient responses only, and in the study by Warner et al<sup>48</sup> none of nine patients responded, and five could not tolerate the treatment.

At least two-thirds of patients attain safe platelet counts (minimum, 30,000/ $\mu$ L) within 1 to 2 weeks of receiving corticosteroids. Most responders achieve platelet counts greater than 100,000 to 150,000/ $\mu$ L. However, ITP in adults usually is a chronic disorder, and most patients have a relapse of thrombocytopenia, either during tapering of prednisone, or within months after stopping the drug.<sup>49</sup>

Overall, approximately 50% of patients have a platelet count above 50,000/ $\mu$ L after stopping prednisone.<sup>40,41,50,51</sup> Patients with shorter duration of bleeding symptoms are more likely to respond favorably.<sup>51</sup> For patients who suffer relapse of thrombocytopenia after initial complete remission, an additional course of prednisone can be effective.<sup>50</sup>

Results of studies with longer follow-up periods indicate that the continuing risk of relapse among adult patients with initial complete remission is substantial. For example, one study<sup>50</sup> showed that only 29% of adult patients who entered complete remission remained in remission 4 years later. In summary, only approximately 10% to 15% of adult patients with ITP have a durable remission following corticosteroid therapy. Consequently, most need either long-term medical treatment or splenectomy.

Adverse effects of prednisone occur in 5% to 20% of patients and include facial swelling, weight gain, and behavioral changes.<sup>22,51</sup> Less common (1% to 5%) but important complications include infection, myopathy, hyperglycemia, psychosis, hypertension, hypokalemia, and osteoporosis. Osteonecrosis, most commonly involving the femoral head, occurs as a late side effect in approximately 5% of patients who undergo prolonged therapy. This complication sometimes occurs after intensive short-term corticosteroid therapy.<sup>52</sup>

### Intravenous Immune Globulin

High-dose IVIG has been used to manage ITP since 1981.<sup>53,54</sup> Prepared by means of ethanol precipitation of pooled plasma

followed by a technique to minimize IgG self-aggregation, monomeric IgG is stabilized with sugar. Thus, more than 95% of IVIG consists of monomeric IgG. The IgG aggregates, IgA, and other contaminants constitute a negligible fraction. The IgG possesses both the Fab and Fc activities required for antigen neutralization and reticuloendothelial cell interaction, respectively, and has the expected half-life and subclass distribution of human IgG.

Reticuloendothelial blockade is the principal mechanism of action of IVIG. Treatment with high-dose IVIG increases serum IgG concentration two- to fourfold, a level that blocks clearance of radiolabeled, IgG-sensitized red cells. Monomeric IgG is in equilibrium with the Fc receptors on the reticuloendothelial cells, and even though the binding affinity for monomeric IgG is low, the high plasma IgG concentration results in high receptor occupancy. However, this reticuloendothelial blockade theory cannot explain the occasional long-term response to IVIG treatment, nor can it account for the observation that Fc-depleted immunoglobulin preparations can be effective in the management of ITP. Thus, other mechanisms of IVIG have been proposed, including the reduction of antibody synthesis; IgG molecules against the Fab regions of pathogenic autoantibodies ("antiidiotypic antibodies")<sup>55</sup>; cytokine-induced pro- or anti-inflammatory effects<sup>56</sup>; up- or downregulation of various FcγRs; or the formation of soluble immune complexes. In a mouse model of ITP, the inhibitory IgG receptor, FcγRIIB, was required for IVIG to cause an elevation in platelet count,<sup>57</sup> and the transfer of IVIG-primed dendritic cells has been shown to recapitulate the effect of IVIG.<sup>58</sup>

The use of IVIG is indicated for patients with ITP with bleeding or for whom an invasive procedure is planned, because it is more effective than corticosteroids at raising the platelet count quickly. A French study of 122 adults with ITP showed that IVIG (0.7 g/kg/day for 3 days) raised the platelet count to over 50,000/μL within 5 days more frequently than did corticosteroids (methylprednisolone, 15 mg/kg/day for 3 days).<sup>59</sup> However, the clinical impact of these observations can be debated.

The usual dose of IVIG is 2 g/kg, given either as 0.4 g/kg for 5 consecutive days or as 1 g/kg over 2 days. One trial showed no difference between one and two doses of 1 g/kg.<sup>60</sup> Thus, one approach is to give 1 g/kg as a single dose and to repeat the dose 2 days later if no significant platelet count increase has occurred.<sup>61</sup> This may avoid unnecessary use of a second dose. To minimize acute side effects, the infusion is given over at least 4 to 8 hours. There is no evidence to suggest that any one preparation of IVIG is superior.

IVIG increases the platelet count to greater than 50,000/μL in approximately 80% of adult patients with chronic ITP, often within 1 to 3 days following start of treatment. In more than half of responders, platelet counts become normal. Unfortunately, responses are usually transient, and tachyphylaxis is common. Platelet counts peak 5 to 10 days after treatment and return to baseline within 2 to 6 weeks.

Although long-lasting complete remission after a single course of IVIG for chronic ITP in adults is uncommon, repeated

treatments separated by a few weeks result in complete or partial remission without need for further IVIG in up to one-third of patients.<sup>60</sup> Of the remaining patients, approximately one-half continue to respond to ongoing maintenance therapy, and one-half experience refractoriness. However, the high cost and time requirement often preclude the use of maintenance IVIG.

IVIG is generally regarded as being safe, although important adverse events are described.<sup>62</sup> Common but mild side effects, which occur among 15% to 75% of patients, include headache, backache, nausea, flushing, and fever.<sup>63</sup> These can be controlled by temporarily stopping or reducing the rate of infusion. Premedication with acetaminophen, antihistamine, or corticosteroids can be helpful. Chest pain, hypertension, hypotension, bronchospasm, and laryngeal edema are uncommon, and other rare side effects include hemolysis caused by ABO alloantibodies within the IVIG, acute renal failure, and aseptic meningitis.<sup>22</sup> Administration of high-dose IVIG can be complicated by thrombotic events, including myocardial infarction, stroke, and deep venous thrombosis (DVT), particularly in elderly patients.<sup>64</sup> Because IVIG is prepared from pooled plasma from thousands of donors, infection transmission is theoretically possible. Transfusion-related acute lung injury following IVIG also has been described.<sup>65</sup>

### Intravenous Rh Immune Globulin

Like IVIG, the mechanism of action of intravenous RhIG is believed to be reticuloendothelial blockade, which probably occurs through occupancy of the reticuloendothelial cell FcγRs by IgG-sensitized red cells. The result is reduced platelet clearance. Thus, the therapy is only effective in D-positive individuals. Further support for this mechanism of action is the observation that plasma containing high-titer anti-c also can increase the platelet count of patients with ITP whose red cells bear this Rh alloantigen. Intravenous RhIG usually is ineffective in the treatment of patients who have undergone splenectomy.<sup>66</sup> Cytokine/chemokine secretion by reticuloendothelial cells may also be an important mechanism for the platelet count rise following treatment with RhIG.<sup>67</sup>

Initially, lower doses of intravenous RhIG were used, but doses of 75 μg/kg have been shown to produce a more rapid increase in platelet count with a longer duration of response.<sup>68</sup> The likelihood of a platelet count response following 75 μg/kg is similar to that of IVIG<sup>69</sup>; likewise, the increase in platelet count usually is transient (2 to 4 weeks), although long-term remissions sometimes occur. The peak platelet count tends to be lower with intravenous RhIG than with IVIG, but the time to reach peak platelet count often is longer; therefore, a greater interval between maintenance injections may be possible with RhIG.<sup>70</sup> Some patients who do not respond to IVIG respond to intravenous RhIG, and vice versa. Costs associated with use of RhIG in the United States are approximately 35% to 60% less than the costs of IVIG.<sup>71</sup>

A clinically important adverse effect of intravenous RhIG is alloimmune hemolysis. Usually, only a minor decrease in

hemoglobin level occurs (less than 20 g/L), although a positive direct antiglobulin test is universal. However, in at least 1 in 1000 recipients, life-threatening acute intravascular hemolysis results, necessitating transfusion or even hemodialysis.<sup>72</sup> Such patients have usually received the US Food and Drug Administration (FDA)-approved dose (50 µg/kg), and the explanation for the severe hemolysis is unknown. Another rare complication is disseminated intravascular coagulation (DIC), with some fatal outcomes reported.<sup>73</sup> Although current intravenous RhIG preparations are considered pathogen-free, the theoretical risk of transmitting infection is illustrated by the epidemic of hepatitis C during 1978 and 1979 in Ireland. It affected young mothers who received perinatal RhIG contaminated with this virus from a single infected donor.<sup>74</sup> Current availability of solvent/detergent-treated RhIG should make the risk of infection negligible. Table 23-1 compares IVIG and intravenous RhIG.

Splenectomy

Splenectomy is frequently successful, because the spleen is the major site of both autoantibody production and platelet destruction. Complete remission occurs in approximately 75% of patients within 4 to 6 weeks after splenectomy.<sup>75</sup> In complete responders, normal platelet counts are reached within 7 days in 90% and within 6 weeks in 98% of patients; spontaneous remission is encountered rarely thereafter. In a further

Table 23-1. Comparison of High-Dose IVIG and Intravenous RhIG for Management of Immune Thrombocytopenic Purpura

| Characteristic                              | High-Dose IVIG                                                                                                    | Intravenous RhIG                                                                                |
|---------------------------------------------|-------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|
| Side effects                                | Common: headache, hypertension, fever and chills<br>Rare: hemolysis, renal failure, myocardial infarction, stroke | Common: mild hemolysis<br>Rare: severe hemolysis necessitating transfusion or hemodialysis, DIC |
| Response rate                               | ~80%                                                                                                              | ~80%                                                                                            |
| Response duration                           | Usually transient                                                                                                 | Usually transient                                                                               |
| Pattern of platelet increase                | Faster increase, higher peak, shorter duration of response                                                        | Slower increase, lower peak, longer duration of response                                        |
| Influence of ABO, Rh type                   | No influence                                                                                                      | Only D-positive patients respond                                                                |
| Influence of splenectomy                    | Unknown                                                                                                           | Minimal response in patients without a spleen                                                   |
| Suitability for emergency management of ITP | Recommended                                                                                                       | Not recommended                                                                                 |

IVIG = intravenous immune globulin; RhIG = Rh immune globulin; DIC = disseminated intravascular coagulation; ITP = immune thrombocytopenic purpura.

5% to 10% of patients, partial remission is achieved. In a systematic review of the efficacy of splenectomy for patients with ITP, younger age was an independent predictor of response to splenectomy<sup>75</sup>; however, other investigators have shown that response to IVIG<sup>76–78</sup> and splenic clearance of platelets (determined by radionuclide platelet imaging techniques)<sup>79</sup> are good predictors of response to splenectomy. The risk of relapse after a complete remission following splenectomy is low (approximately 10% to 15% at 10 years).

Perioperative management of splenectomy involves 1) optimization of the platelet count before surgery; 2) vaccination to minimize the risk of overwhelming postsplenectomy infection; and 3) prevention of thromboembolism. Adequate perioperative hemostasis usually can be achieved with preoperative high-dose IVIG or corticosteroids. Sometimes despite these measures, patients have thrombocytopenia at splenectomy. However, most patients do not have excessive bleeding; therefore, platelet transfusions should be reserved for perioperative bleeding.

Laparoscopic splenectomy is an increasingly popular surgical approach, with outcomes similar to open splenectomy.<sup>80,81</sup> A concern is that residual spleen tissue (accessory spleens) may be missed more frequently than with open splenectomy; however, this did not occur in a recent large cohort study.<sup>81</sup>

The presence of residual accessory spleen should be considered when splenectomy fails, or when patients relapse months or years after surgery.<sup>82</sup> Postsplenectomy blood film changes (eg, Howell-Jolly bodies) do not necessarily eliminate the possibility of a residual accessory spleen. Approximately 10% to 50% of patients with postsplenectomy relapse have accessory spleens detected with sensitive radionuclide imaging, such as heat-damaged red cells labeled with technetium-99m or platelets labeled with indium-111; however, a durable platelet count response following accessory splenectomy performed because of postsplenectomy relapse of ITP is expected in less than 50% of patients.

Perioperative morbidity after splenectomy is less than 10%. The most frequent complications are pleuropulmonary (pneumonia, subphrenic abscess, pleural effusion) in 4% of patients, major bleeding in 1.5%, and thromboembolism in 1%. Morbidity is probably even lower when laparoscopic splenectomy is performed by an experienced surgeon. Overall mortality is approximately 1% following laparotomy and 0.2% following laparoscopic splenectomy.<sup>75</sup>

The major long-term risk is overwhelming postsplenectomy infection. In a pooled analysis of 19,680 splenectomized patients from 78 studies, the incidence of postsplenectomy septicemia and/or meningitis (median follow-up, 6.9 years) was 3.2%, with a 44% case-fatality rate (overall infection-associated mortality rate, 1.4%).<sup>83</sup> The incidence of septicemia/meningitis and infection-associated mortality was 2.1% and 1.2%, respectively, among the 484-patient subgroup splenectomized because of ITP.<sup>83</sup> To reduce this risk, all patients should be vaccinated with polyvalent pneumococcal vaccine, quadrivalent meningococcal polysaccharide vaccine, and *Haemophilus influenzae* type b vaccine, preferably at least 2 weeks before splenectomy.<sup>84</sup>

Postoperative thromboembolism is the most common cause of postoperative mortality; thus, for patients with delayed postoperative mobilization, prophylactic low-molecular-weight (LMW) heparin should be considered when the platelet count is recovering.

### Long-Term Management: Second-Line Therapy

About 10% to 15% of patients with ITP do not attain a durable response despite prednisone and splenectomy. The large number of therapies that have been used for these treatment failures attests to the difficulty in caring for such patients. In a systematic review, rituximab, azathioprine, and cyclophosphamide had the most reported complete responses in such refractory patients.<sup>85</sup>

#### Rituximab

Rituximab is a chimeric monoclonal anti-CD20 currently indicated for the treatment of lymphoma and rheumatoid arthritis. The Fab portion binds to CD20 on B lymphocytes, which results in Fc $\gamma$ R-mediated B-cell lysis by complement-dependent<sup>86</sup> and antibody-dependent pathways.<sup>87-89</sup> In ITP, rituximab depletes CD20-positive B cells, which are responsible for platelet autoantibody production. Rituximab-coated B cells may also cause reticuloendothelial blockade, possibly accounting for the rapid response to treatment observed in some patients.<sup>90</sup> Recently, platelet count responses to rituximab have been correlated with the normalization of T-cell abnormalities in ITP patients, suggesting that rituximab may be more effective at an early stage of ITP, when T-cell expansion is still dependent on B-cell co-stimulation.<sup>91</sup>

A systematic review<sup>92</sup> of the literature described outcomes in adults who had ITP for 1 to 360 months (half after splenectomy). A complete response to rituximab (platelet count greater than 150,000/ $\mu$ L) was seen in 43.6% of patients (95% CI, 29.5% to 57.7%) and an overall response (platelet count greater than 50,000/ $\mu$ L) in 62.5% (95% CI, 52.6% to 72.5%). Responses lasted a median of 10 months. Approximately one-third of patients treated with rituximab may achieve a durable remission.<sup>93</sup> Results with rituximab in children are less encouraging. In a prospective single-arm study of 36 children with chronic ITP treated with rituximab, 11 (30.6%) achieved a platelet count of 50,000/ $\mu$ L or greater for 4 consecutive weeks.<sup>94</sup>

Rituximab is generally well tolerated. However, infusional side effects, including chills, blood pressure fluctuations, and respiratory complaints, occur in approximately 20% of patients with ITP. Serum sickness has also been reported.<sup>94</sup> In 2006, the FDA issued a safety alert following two fatal cases of progressive multifocal leukoencephalopathy (a rare neurologic syndrome caused by JC virus reactivation) among patients with SLE treated with rituximab.<sup>95</sup>

#### Danazol

Danazol is an attenuated androgen with mild virilizing effects that can be used to treat men and nonpregnant women with ITP. Its mechanism of action is to decrease Fc $\gamma$ R numbers and the rate of Fc $\gamma$ R-mediated clearance of IgG-sensitized cells. Danazol decreases the number of monocyte IgG Fc receptors.<sup>96</sup> Usually, 400 to 800 mg is administered daily in divided doses, although

some physicians use low doses (50 mg/day). A response usually occurs within 2 months, although when low doses are used, as long as 6 months may be needed.

Danazol appears to produce a sustained increase in platelet count in approximately 30% to 40% of treated patients. The response rate may be higher among patients with associated rheumatologic disorders.<sup>97</sup> Danazol must be continued to maintain the platelet count response, although attempts at dose reduction should be made.

Danazol is generally well tolerated. The most frequent adverse effects are mild virilization, fluid retention, nausea, amenorrhea, and liver dysfunction. Peliosis of the liver and spleen, headaches, increased fibrinolysis, and idiosyncratic thrombocytopenia are rare side effects. Fetal damage precludes use during pregnancy.

#### Vinca Alkaloids

Vinca alkaloids (vincristine, vinblastine) can produce generally short-lived increases in platelet count in approximately 65% of patients.<sup>98</sup> These drugs bind to platelet microtubules and so might work by being delivered to, and thereby inhibiting, reticuloendothelial macrophages. Repeated doses of vinca alkaloids are rarely used to treat patients with chronic ITP, mostly because of dose-dependent neuropathy.

#### Immunosuppressive Agents

Cyclophosphamide, azathioprine, mycophenylate, and cyclosporine are immunosuppressive agents that have been used to treat patients with refractory ITP. Cyclophosphamide is an alkylating agent given in doses of 1 to 2 mg/kg/day, although high-dose cyclophosphamide has been used in combination with autologous stem cell transplantation.<sup>99</sup> Azathioprine and mycophenylate are purine antimetabolites that inhibit lymphocyte proliferation. Cyclosporine is a calcineurin inhibitor that selectively blocks T-cell-dependent biosynthesis of lymphokines, particularly interleukin-2, at the level of messenger RNA transcription.

These drugs, alone or in combination, have shown moderate success, with up to two-thirds of patients exhibiting a platelet count response.<sup>85,100,101</sup> However, only 20% to 30% have responses that persist after stopping the drug(s). Responses may occur as early as 2 weeks and as late as 3 months after initiation of treatment.

Leukopenia is a limiting side effect of azathioprine and occasionally mycophenylate, and the concern over leukemic transformation has limited use of cyclophosphamide.<sup>102</sup> Animal studies suggest that intermittent cyclophosphamide may be less leukemogenic than daily administration. Cyclophosphamide also can cause hemorrhagic cystitis and hepatic toxicity. Cyclosporine is associated with hypertension and renal failure in some patients.

#### *Helicobacter pylori* Eradication

Molecular mimicry between microbial antigens and platelet antigens is the proposed mechanism of ITP in the setting of *H. pylori* infection.<sup>103</sup> *H. pylori* can be successfully eradicated in 87% of patients with a 1- to 2-week course of antibiotics and proton



pump inhibitors, with some long-term remissions of concomitant ITP reported. Further, in a recent meta-analysis of 788 patients (half of whom had received prior treatment with corticosteroids, immunosuppressive agents, or splenectomy), *H. pylori* treatment and eradication were each associated with platelet counts that were 34,000/ $\mu$ L and 52,000/ $\mu$ L higher, respectively, than untreated and noneradicated controls.<sup>104</sup> Although this meta-analysis helps to support a pathogenic role for *H. pylori* infection in ITP, the conflicting results among individual studies remain unexplained.

### Thrombopoietin Agonists

The recent success of TPO agonists in patients with ITP has reinforced the concept of platelet underproduction as an important mechanism of thrombocytopenia in ITP. TPO agonists activate the c-Mpl receptor on megakaryocytes, thereby stimulating platelet production. Unlike recombinant TPO, these molecules do not resemble endogenous TPO, and thus do not stimulate formation of TPO-reactive autoantibodies. Subcutaneous and oral preparations have been developed, with both showing promising results in Phase I and II studies.<sup>105,106</sup> A platelet count increase during continuing TPO agonist administration is achieved in 60% to 75% of patients. Mild-to-moderate increase in marrow reticulin (but without collagen fibrosis and with normal cytogenetic findings) was observed in two patients.<sup>105</sup>

The precise role for TPO agonists in the treatment of ITP is still uncertain, and full reports of Phase III trials are anticipated. These agents may be most appropriate for refractory patients after splenectomy or to prepare for an invasive procedure. The potential to spare patients the need for splenectomy is also under investigation.

### Other Treatments

Other agents that have been used for patients with refractory ITP include colchicine, dapsone, interferon- $\alpha$ , protein A immunoadsorption, and plasmapheresis. Interferon- $\alpha$ -2b may be effective for patients with hepatitis C-associated thrombocytopenia.<sup>107</sup> Blocking monoclonal antibodies against the Fc $\gamma$ R<sup>108</sup> and against CD40 ligand (CD154)<sup>109</sup> have also shown promise in selected patients with refractory disease.

### Special Treatment Situations

#### Emergency Treatment of a Bleeding Patient

Any patient with ITP who has life-threatening bleeding needs immediate platelet transfusion (5 to 10  $\times 10^{11}$  platelets, or approximately 5 to 10 units). The patient then should receive IVIG (1 g/kg) over 4 to 6 hours to block the reticuloendothelial system, and additional platelet transfusions as required.<sup>110</sup> Some physicians use continuous infusions of IVIG and platelets for this situation.<sup>111</sup> Other treatments (eg, corticosteroids) to achieve longer-term control of thrombocytopenia should be started simultaneously.

#### Preparation for Invasive Procedures

High-dose IVIG usually is the treatment of choice for severely thrombocytopenic patients with ITP who need urgent surgery

or an invasive procedure. Prophylactic platelet transfusion should be administered only when maximal hemostasis is required, because transfusion-induced platelet count increments are usually transient. For situations in which at least 2 or 3 days are available before the planned procedure, less expensive and equally effective options include corticosteroids or intravenous RhIG.

### Immune Thrombocytopenic Purpura in Pregnancy

Thrombocytopenia during pregnancy usually is not caused by ITP. Rather, a benign condition known as *gestational thrombocytopenia* is more common, occurring in approximately 5% of pregnancies at term. In this condition, maternal platelet counts do not fall below 70,000/ $\mu$ L.<sup>112</sup> Newborns of mothers with incidental thrombocytopenia are not at increased risk of neonatal thrombocytopenia.<sup>113</sup> The second most likely cause of thrombocytopenia is pregnancy-related disorders [eg, preeclampsia and syndrome of hemolysis, elevated liver enzymes, and low platelet count (HELLP syndrome)]. Preeclampsia occurs in approximately 10% of pregnancies and causes thrombocytopenia in one-fourth of affected mothers. Preeclampsia is associated with increased maternal and fetal morbidity and mortality. Secondary causes of immune thrombocytopenia that can occur in young women, such as SLE or HIV infection, should also be considered in the appropriate clinical context.

ITP in pregnancy is an important disorder because of the treatment implications for the mother and the possibility of fetal or neonatal thrombocytopenia caused by transplacental passage of IgG platelet autoantibodies. In the past, infants of mothers with ITP were delivered by cesarean section; however, today, this procedure is not routinely recommended, for two reasons. First, the frequency of severe fetal thrombocytopenia (platelet count less than 20,000/ $\mu$ L) is low (approximately 4%).<sup>114</sup> Second, there is no evidence that cesarean section leads to less intracranial bleeding compared with vaginal delivery.

Many pregnant women with ITP do not need specific treatment, unless the platelet count decreases below 20,000/ $\mu$ L or there is evidence of impaired hemostasis. The two preferred treatment options are intermittent high-dose IVIG and low-dose prednisone; however, IVIG is generally the treatment of choice (1 g/kg every 2 to 4 weeks) because side effects are more common with prednisone and include teratogenicity (first trimester) and preeclampsia (second and third trimesters). At delivery, thrombocytopenic patients should not be subjected to epidural analgesia. Platelet transfusions are almost never needed at delivery. In the largest cohort of infants born to mothers with ITP (119 pregnancies in 97 women), 10% of infants had a platelet count below 50,000/ $\mu$ L at birth, 15% required hemostatic treatments, and 1% died.<sup>115</sup>

There are no good predictors of fetal thrombocytopenia, not even the severity of maternal thrombocytopenia (indeed, women cured of ITP by splenectomy can bear infants with passive autoimmune thrombocytopenia). Results of maternal platelet-antibody tests also are unhelpful. Although it is

possible to determine the fetal platelet count by fetal cord sampling (cordocentesis), this procedure causes morbidity or mortality in 1% to 2% of fetuses sampled. Consequently, this procedure is generally discouraged.

At the authors' facility, pregnant patients with ITP are followed with platelet counts every few weeks. Testing for antiphospholipid antibodies (lupus anticoagulant and anticardiolipin antibodies) is performed because this can be an indication for treatment, especially if previous miscarriages have occurred.<sup>116</sup> If therapy for maternal thrombocytopenia is needed, IVIG is preferred. Splenectomy is rarely indicated for severe, refractory thrombocytopenia in pregnancy.<sup>22</sup>

Unless cesarean section is needed for obstetric indications, spontaneous vaginal delivery is recommended for mothers with ITP. An immediate fetal umbilical vein platelet count is obtained at delivery. Relatively severe neonatal thrombocytopenia (platelet count less than 50,000/ $\mu$ L) should be aggressively managed with IVIG or corticosteroids. It is important to recognize that the platelet count of a newborn infant with passive autoimmune thrombocytopenia may decrease for up to 5 days after delivery,<sup>117</sup> possibly because of the recruitment of reticuloendothelial cells within the lung. A daily platelet count should be obtained until the platelet count normalizes.

### Acute Immune Thrombocytopenic Purpura of Childhood

Acute ITP of childhood is a relatively common, generally self-limited, immune thrombocytopenic disorder with a peak incidence between 2 and 6 years of age. Boys and girls are equally affected, except for infants, in which males predominate.<sup>118</sup> Most children (80% to 90%) with acute ITP recover completely within 6 months; the others have persistent thrombocytopenia. Acute ITP of childhood likely is a transient autoimmune disorder.<sup>119</sup>

The typical clinical manifestations are bleeding and bruising following a viral infection. The mortality is approximately 0.4% and most deaths are caused by intracranial hemorrhage. Laboratory abnormalities include isolated thrombocytopenia and normal or increased MPV. A marrow examination usually is not performed on a child with typical clinical features of ITP, but is indicated for unexpected clinical or laboratory findings. Normal or increased numbers of megakaryocytes are observed. Sometimes, morphologically distinct lymphoid cells, called *hematogones*, constitute up to one half of the marrow cells and may cause confusion with acute leukemia.<sup>120</sup> These nonneoplastic cells have the surface immunophenotypic profile of immature lymphocytes.

Acute ITP in children usually is a benign disease. Although rare, intracranial hemorrhage is the most feared complication. Clinical trials have focused on the time to increase the platelet count to safe levels as a surrogate endpoint for avoiding intracranial hemorrhage.

In a meta-analysis of six randomized trials ( $n = 410$  children), IVIG was associated with a more rapid platelet count increase compared with corticosteroids.<sup>121</sup> On the basis of these results, high-dose IVIG is generally recommended for initial treatment of severe acute ITP in children. Oral prednisone or an additional dose of IVIG is added if the platelet count remains below 20,000/ $\mu$ L by 48 hours. It appears that lower doses of IVIG (eg, 250 to 500 mg/kg/day for 2 days rather than the standard 1 g/kg/day) may also be effective.<sup>122</sup>

High-dose corticosteroid therapy (eg, intravenous methylprednisolone at 30 mg/kg/day for 3 days; or oral methylprednisolone 30 to 50 mg/kg/day for 7 days) also rapidly increases the platelet count in children with acute severe ITP.<sup>123</sup> Combined treatment with IVIG and pulse methylprednisolone may be indicated for those children felt to be at very high risk of intracranial hemorrhage.<sup>124</sup>

### Chronic Immune Thrombocytopenic Purpura of Childhood

Approximately 10% to 20% of children with ITP eventually have chronic ITP, defined as a platelet count less than 100,000/ $\mu$ L for more than 6 months. As many as one-third of children who meet this definition can still enter late spontaneous remission, sometimes as long as 5 to 10 years after diagnosis. Chronic ITP in children resembles adult chronic ITP.

Some patients have no symptoms despite marked thrombocytopenia. These children generally do not need treatment. For children with symptoms, options include long-term or intermittent administration of corticosteroids, maintenance IVIG or RhIG, splenectomy, vinca alkaloids, and immunosuppressive agents.<sup>125</sup>

Intravenous immune globulin (2 g/kg over 2 to 5 days) increases the platelet count of most children with chronic ITP<sup>53,125</sup> and repeated courses of IVIG have been used to defer or avoid splenectomy. This practice also appears to be cost-effective.<sup>126</sup> Unfortunately, approximately 25% of patients become refractory. Low-dose, alternate-day corticosteroid therapy may improve the results with maintenance IVIG.

Intravenous RhIG is effective in increasing the platelet count of most Rh-positive children with chronic ITP<sup>70,127</sup> and, at a dose of 75  $\mu$ g/kg, appears to be as effective as IVIG.<sup>69</sup> The benefit is transient and the median duration of benefit is approximately 3 weeks. RhIG can also be considered splenectomy-sparing in some patients.<sup>127</sup> However, RhIG is generally ineffective following splenectomy.<sup>127</sup>

Splenectomy is sometimes performed in children with chronic ITP who cannot be maintained on low-dose maintenance glucocorticoids, IVIG, or RhIG therapy. Because of the high probability of a cure (approximately 70%), some physicians perform splenectomy before instituting potentially toxic immunosuppressive therapy. However, in general, splenectomy is avoided in children, especially young children, because of the

high risk of postsplenectomy sepsis, the possibility of late spontaneous remissions, and the efficacy of intermittent maintenance therapy with IVIG or RhIG. Patients must receive polyvalent pneumococcal, meningococcal, and *H. influenzae* type b vaccination, at least 2 weeks before the splenectomy.<sup>84</sup>

Results of use of immunosuppressive agents (azathioprine or cyclophosphamide) and vinca alkaloids to treat children with chronic ITP are variable and based on uncontrolled studies.<sup>125</sup> Pulse corticosteroids have been used in children, although few durable remissions occur. In one study, three of seven children achieved a platelet count above 50,000/ $\mu$ L 6 months after receiving high-dose dexamethasone. One child had a response that was maintained to 1 year.<sup>128</sup> Children with chronic ITP refractory to splenectomy occasionally obtain benefit from cyclosporine. Danazol can be tried in the adolescent patient. About one-third of pediatric patients with chronic ITP responded to rituximab in one study.<sup>94</sup>

## Secondary Immune Thrombocytopenic Purpura

### Systemic Lupus Erythematosus and Antiphospholipid Antibody Syndromes

SLE and antiphospholipid antibody syndrome (APLAS) are autoimmune disorders complicated by thrombocytopenia in 15% to 25% of patients. Mechanisms of thrombocytopenia include platelet glycoprotein-reactive autoantibodies,  $\beta_2$ -glycoprotein 1-containing and other platelet-reactive immune complexes, and hypersplenism, among others. Whether antibody-induced platelet activation in vivo contributes to thrombosis in SLE or APLAS is uncertain.

Various thrombocytopenic syndromes occur. Sometimes the thrombocytopenia is chronic, resembling ITP, and is the predominant clinical manifestation of SLE or APLAS.<sup>129</sup> Sometimes, patients with even mild thrombocytopenia have platelet dysfunction and a prolonged bleeding time. Thrombocytopenia complicated by antiphospholipid antibodies carries increased risk of thrombosis, rather than bleeding.<sup>130</sup> These patients often have livedo reticularis or acrocyanosis that can progress to digital necrosis.<sup>131</sup> Acute, severe thrombocytopenia can be prominent in patients with severe multisystem exacerbation of SLE<sup>129</sup> or the catastrophic APLAS.<sup>132</sup> In rare instances, patients with SLE have an illness that closely resembles thrombotic thrombocytopenic purpura (TTP) or hemolytic uremic syndrome (HUS). These patients may require plasma exchange.<sup>133</sup>

Thrombocytopenia associated with SLE is managed similarly to ITP, except that danazol should be tried before splenectomy. Antiplatelet agents are recommended in patients with APLAS as soon as a hemostatic platelet count is achieved; in patients who have thrombotic complications, full-dose anticoagulation is required. Corticosteroids are used as initial therapy, although thrombocytopenia may recur during tapering of prednisone. Steroid maintenance should be considered especially if this

treatment controls other aspects of SLE. The role of corticosteroids in APLAS-associated thrombocytopenia is less clear. Some patients develop progressive ischemic necrosis of digits during corticosteroid therapy.<sup>134</sup>

Splenectomy is probably as effective in achieving platelet count remission in SLE and APLAS as it is in ITP.<sup>135</sup> Some physicians first try danazol (an attenuated androgen) for several weeks or months in doses of 200 to 1200 mg/day<sup>97</sup> before splenectomy. For patients with disease that is refractory to danazol and splenectomy, more aggressive therapies such as high-dose IVIG, rituximab, azathioprine, mycophenylate, intermittent pulse cyclophosphamide,<sup>136</sup> plasmapheresis synchronized with pulse intravenous cyclophosphamide,<sup>137</sup> and cyclosporine<sup>138</sup> can be tried.

### HIV-Associated Immune Thrombocytopenic Purpura

Thrombocytopenia occurs in approximately 10% to 15% of persons with asymptomatic HIV infection and 30% to 40% of patients with AIDS. Besides immune platelet destruction, there are many other ways that infection with HIV can cause thrombocytopenia,<sup>139</sup> including impaired platelet production secondary to HIV infection of megakaryocytes, drug-induced myelosuppression (commonly, zidovudine, ganciclovir, and trimethoprim/sulfamethoxazole), HIV-associated thrombotic microangiopathy, hypersplenism, and marrow infiltration by tumor or opportunistic infections. Platelet kinetic studies have shown a complex interaction of impaired platelet production, increased platelet destruction, and predominant splenic platelet sequestration and destruction.<sup>140</sup>

Immune platelet destruction may be related to antibodies that cross-react with polymorphic regions on HIV-1 proteins and GPIIb/IIIa or GPIb/IX complexes.<sup>141</sup> Immune complexes containing IgM anti-idiotypic antibodies against anti-GPIIIa may explain the paradox of high levels of platelet-bound IgG and IgM but low levels of serum platelet-reactive antibodies in HIV-associated ITP.<sup>142</sup>

The occurrence of isolated immune thrombocytopenia does not necessarily indicate an increased risk of progression to a more advanced stage of HIV infection. Particularly in children, patients with HIV-associated thrombocytopenia may not even have helper-T-cell lymphopenia. Sometimes thrombocytopenia resolves spontaneously.

Anti-HIV therapy, as with zidovudine, often increases the platelet count in patients with HIV-associated thrombocytopenia.<sup>143</sup> For patients whose condition is refractory to zidovudine, interferon- $\alpha$  has been reported to be effective. Highly active antiretroviral therapy, typically consisting of two nucleoside analogs in conjunction with an HIV protease inhibitor or a nonnucleoside reverse transcriptase inhibitor, has improved survival among patients with HIV infection. Such regimens also increase the platelet count of patients with thrombocytopenia in inverse proportion to the reduction in HIV viral load.<sup>144</sup>

Many patients have no or minimal bleeding and do not need specific therapy for HIV-associated thrombocytopenia. For patients with symptomatic thrombocytopenia, however, the treatment approach is similar to therapy for classical ITP,<sup>54,70,145</sup> although intravenous RhIG may be somewhat more effective than IVIG in some patients with HIV-associated ITP.<sup>146</sup> Splenectomy appears to be at least as effective in achieving long-term remission of HIV-associated thrombocytopenia as it is in the management of primary ITP.<sup>147</sup> Splenectomy also does not accelerate, and may even reduce, the rate of progression to AIDS among persons with HIV infection.

### Neoplasia-Associated Immune Thrombocytopenic Purpura

Immune thrombocytopenia can precede or accompany neoplastic disorders, especially lymphoproliferative syndromes.<sup>148</sup> Decreased platelet survival and the presence of platelet-reactive autoantibodies<sup>149</sup> have been found. In general, the thrombocytopenia represents less of a risk to the patient than does the underlying malignant disease; thus, the first step is management of the underlying disorder. Sometimes thrombocytopenia resolves as the tumor responds to treatment, but at other times, it necessitates therapy similar to chronic ITP (corticosteroids, splenectomy, etc). In one study, 30% of patients with ITP and Hodgkin disease responded to corticosteroids, whereas 75% benefited from splenectomy.<sup>148</sup>

### Transplantation-Associated Immune Thrombocytopenic Purpura

Severe persistent thrombocytopenia despite recovery of red and white cells is relatively common after allogeneic or autologous hematopoietic stem cell transplantation (HSCT). Autoimmune thrombocytopenia has been implicated in some patients. Late-onset thrombocytopenia following HSCT or solid-organ transplantation that responds to glucocorticoids, IVIG, and splenectomy also has been attributed to autoimmune and even alloimmune thrombocytopenia.<sup>150</sup> However, in any consideration of immune-mediated thrombocytopenia, more common explanations for thrombocytopenia should be suspected including infection, organ rejection, and drugs (eg, vancomycin,<sup>151</sup> azathioprine, sulfamethoxazole-trimethoprim, etc). Approximately 2% of recipients of solid-organ transplants have microangiopathic hemolysis within the first year after transplantation.<sup>152</sup>

## Drug-Induced Immune Thrombocytopenia

Drugs can produce several immune-mediated thrombocytopenic disorders<sup>4,153–156</sup> (Table 23-2). The most common is HIT, which paradoxically is associated with increased risk of thrombosis but not bleeding. In contrast, many other drugs in rare instances cause severe thrombocytopenia and bleeding, a

syndrome called *drug-induced immune thrombocytopenic purpura* (DITP) to emphasize its clinical resemblance to acute ITP. Some drugs cause atypical clinical signs and symptoms (eg, abciximab-induced thrombocytopenia) or trigger an illness that resembles TTP or HUS.

### Heparin-Induced Thrombocytopenia

Heparin-induced thrombocytopenia is a relatively common, IgG-mediated, adverse reaction to heparin that has a strong association with venous and arterial thrombosis.<sup>2-4,157</sup> Heparin-induced thrombocytopenia can be considered a clinicopathologic syndrome; that is, the diagnosis is made most reliably on both clinical and serologic grounds.<sup>158,159</sup> Thus, HIT antibody formation without thrombocytopenia or other abnormalities is not HIT, whereas HIT antibody formation accompanied by an otherwise unexplained 50% or greater postoperative decrease in platelet count (even if the platelet count remains greater than 150,000/ $\mu$ L)<sup>3</sup> or complicated by necrotizing skin lesions at heparin injection sites<sup>157</sup> are examples of HIT syndrome. Indeed, the thrombocytopenia usually is much less severe in HIT<sup>4</sup> than in DITP<sup>160</sup> or GPIIb/IIIa antagonist-induced thrombocytopenia<sup>161</sup> (Fig 23-3). Another contrast from DITP is that even when severe thrombocytopenia occurs in HIT, petechiae and other types of bleeding typically are not observed.<sup>157,162</sup> Indeed, even the one characteristic (although uncommon) hemorrhagic complication of HIT—bilateral adrenal hemorrhage—is caused by thrombosis (adrenal vein thrombosis leading to hemorrhagic necrosis).<sup>157</sup> HIT is an antibody-mediated disorder, and a minimum of 5 days is required for an immunizing exposure to heparin to generate sufficient levels of antibodies to cause thrombocytopenia.<sup>157</sup> Sometimes, onset of thrombocytopenia and thrombosis begins after all heparin has been stopped (“delayed-onset HIT”).

The target antigen recognized by HIT antibodies consists of a multimolecular complex between PF4 (a platelet  $\alpha$ -granule protein of the CXC family of chemokines) and heparin.<sup>15</sup> The HIT antibodies bind to one or more PF4 regions that have undergone conformational modification through binding to heparin. The formation of the antigen is somewhat nonspecific, because PF4 can be rendered antigenic by binding to certain other polyanions, such as pentosan polysulfate or polyvinylsulfonate.<sup>16</sup> At least 12 to 14 saccharide units are needed for heparin to form the antigen complex with PF4. This may explain why LMW heparin preparations are less immunogenic than unfractionated heparin and are less likely to cause HIT.<sup>2,3</sup> Although LMW heparin sometimes causes HIT, it is likely that very small heparin preparations (eg, fondaparinux, a synthetic anti-Factor Xa-binding pentasaccharide) or specially engineered heparins (eg, highly-sulfated heparin moieties bridged with nonsulfated spacer regions) cause HIT rarely, if at all.<sup>163</sup>

Figure 23-4 illustrates several possible mechanisms to explain the intense thrombin generation that occurs in HIT.<sup>164,165</sup> These

Table 23-2. Drug-Induced Immune Thrombocytopenic Syndromes

| Syndrome and Drug(s)                                                                                                                                                                                                         | Comment                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Heparin-induced thrombocytopenia</b>                                                                                                                                                                                      | Prothrombotic reaction caused by heparin-dependent platelet-activating IgG antibodies that recognize platelet factor 4/heparin complexes; caused less often by low-molecular-weight heparin and fondaparinux than by unfractionated heparin                                                                                                                                                                                                                                                                                                              |
| <b>Drug-induced immune thrombocytopenic purpura (DITP)</b>                                                                                                                                                                   | Prohemorrhagic reaction caused by IgG antibodies that recognize drug (or drug metabolite) bound to platelet glycoprotein (GP); patients have severe thrombocytopenia and mucocutaneous bleeding.                                                                                                                                                                                                                                                                                                                                                         |
| <ul style="list-style-type: none"><li>• Quinine</li><li>• Quinidine</li><li>• Rifampin</li><li>• Sulfa antibiotics</li><li>• Vancomycin</li><li>• Iodinated contrast</li><li>• Acetaminophen</li><li>• Many others</li></ul> | <p>Quinine-dependent anti-GPIIb/IIIa and GPIb/IX IgG implicated; drug is widely available (eg, tonic water)</p> <p>Antibodies usually distinct from quinine-dependent antibodies</p> <p>Rifampin-dependent anti-GPIIb/IIIa and GPIb/IX IgG implicated</p> <p>Occurs in ~1 of 25,000 patients receiving trimethoprim-sulfamethoxazole</p> <p>Vancomycin-dependent anti-GPIIb/IIIa IgG</p> <p>Severe thrombocytopenia begins after radiologic procedure</p> <p>IgG recognizes metabolite of acetaminophen</p> <p>See published lists<sup>153,154</sup></p> |
| <b>Atypical DITP</b>                                                                                                                                                                                                         |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
| <ul style="list-style-type: none"><li>• Abciximab</li><li>• Gold</li><li>• MMR vaccine</li></ul>                                                                                                                             | <p>Abrupt onset of severe thrombocytopenia (platelet count nadir, 15,000 to 35,000/<math>\mu</math>L) perhaps by naturally occurring ligand-induced binding site antibodies</p> <p>Thrombocytopenia can persist for months after gold therapy is stopped</p> <p>Transient autoimmune thrombocytopenia (anti-GPIIb/IIIa) that occurs a few weeks after vaccination (resembles childhood acute ITP)</p>                                                                                                                                                    |
| <b>DITP: hapten mechanism</b>                                                                                                                                                                                                | Indicates that IgG recognizes drug that remains bound to platelet surface even following platelet washing                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| <ul style="list-style-type: none"><li>• Penicillin</li></ul>                                                                                                                                                                 | Not well-established                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
| <b>Drug-induced TTP/HUS</b>                                                                                                                                                                                                  |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
| <ul style="list-style-type: none"><li>• Ticlopidine</li><li>• Clopidogrel</li><li>• Quinine</li><li>• Cyclosporine</li><li>• Others</li></ul>                                                                                | <p>Estimated frequency, 1/2,000 to 1/5000</p> <p>Estimated frequency, 1/20,000</p> <p>Quinine-dependent IgG against platelets and other cells found</p> <p>May be pathogenic factor in transplantation-associated TTP/HUS</p> <p>See text</p>                                                                                                                                                                                                                                                                                                            |

MMR = measles-mumps-rubella; ITP = immune thrombocytopenic purpura; TTP = thrombotic thrombocytopenic purpura; HUS = hemolytic uremic syndrome.

include the formation of procoagulant, platelet-derived micro-particles<sup>166</sup> that result from cell signaling triggered by clustering of platelet Fc $\gamma$ Rs. Recent data suggest that in-situ formation of IgG-PF4/heparin complexes on the platelet surface leads to platelet activation.<sup>167</sup> In-vivo platelet activation is suggested by expression of P-selectin by circulating platelets in HIT as well as by increased levels of circulating microparticles. Tissue factor expression by endothelium or monocytes activated by HIT antibodies that recognize PF4 bound to surface glycosaminoglycans are other possible procoagulant events.

Marked in-vivo thrombin generation helps explain several clinical features of HIT, including its association with venous and arterial thrombosis (hypercoagulable state), the occurrence of decompensated DIC with low fibrinogen levels in approximately 10% of patients, and the potential for DVT to progress to venous limb gangrene, particularly in patients treated with warfarin.<sup>165</sup> This last syndrome results from impaired procoagulant-anticoagulant balance: warfarin-induced protein C depletion leads to microvascular thrombosis caused by ongoing intense thrombin generation. Patients with warfarin-induced venous gangrene

typically have a supratherapeutic international normalized ratio (INR), usually more than 4.0. The explanation is a concomitant severe decrease in Factor VII that parallels the decrease in protein C. The importance of in-vivo thrombin generation in HIT provides a rationale for current consensus recommendations that an agent that reduces thrombin generation or directly inactivates thrombin be used for the management of this syndrome.<sup>158,159</sup>

The frequency of HIT varies among different patient populations. Medical patients appear to develop HIT less often than do surgical patients, and female gender is a minor risk factor for HIT.<sup>168</sup> The frequency of HIT can be as high as 5% when multiple risk factors are present (thromboprophylaxis with unfractionated heparin administered for 2 weeks to a female patient following major surgery), whereas the risk is very low or negligible in other settings (eg, administration of LMW heparin during pregnancy).<sup>2,3,169</sup>

**Iceberg Model of HIT**

Figure 23-5 illustrates the relation between clinical HIT and detectability of PF4/heparin antibodies either by washed platelet

**Figure 23-3.** Platelet count nadirs ( $\log_{10}$  scale) and clinical profile of classic drug-induced immune thrombocytopenic purpura (DITP) and heparin-induced thrombocytopenia (HIT). Classic DITP (eg, caused by quinine, vancomycin, or glycoprotein IIb/IIIa antagonists, among many others) typically produces severe thrombocytopenia (platelet count nadir,  $\sim 10,000/\mu\text{L}$ ) and associated mucocutaneous bleeding. In contrast, HIT typically results in mild to moderate thrombocytopenia (median platelet count nadir,  $\sim 60,000/\mu\text{L}$ ) and associated venous or arterial thrombosis. Note that the relative heights of the two peaks are not drawn to scale; HIT is much more common than all other causes of DITP combined. (Used with permission from Warkentin.<sup>4</sup>)

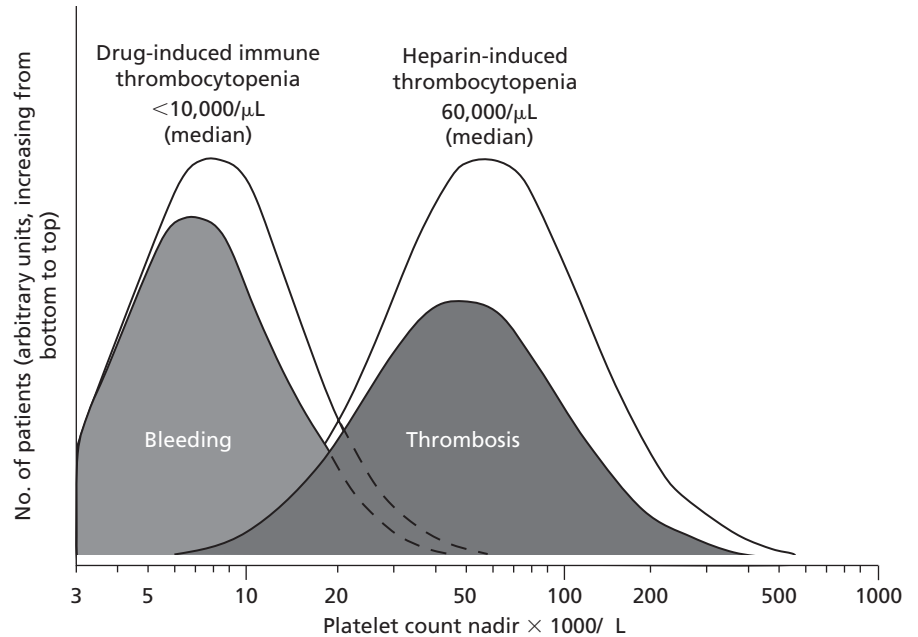
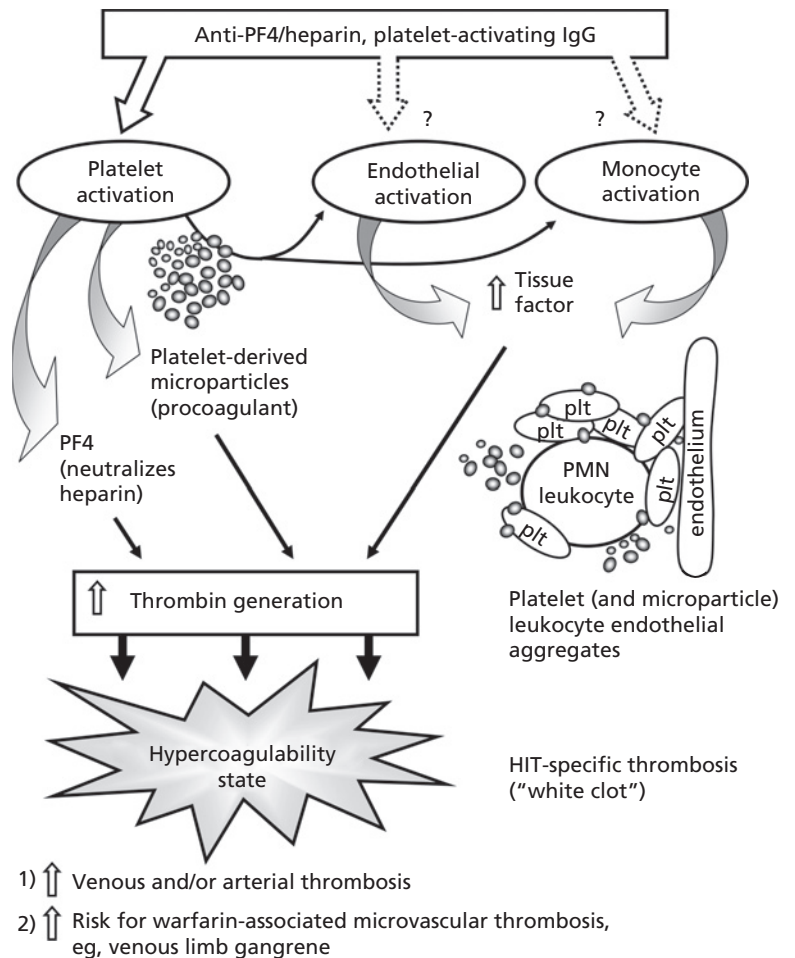


**Figure 23-4.** Pathogenesis of heparin-induced thrombocytopenia. Two explanations for thrombosis in HIT are presented. Activation of platelets by platelet factor 4 (PF4)/heparin IgG antibodies leads to formation of procoagulant, platelet-derived microparticles. Together with neutralization of heparin by PF4 released from activated platelets, there results a marked increase in thrombin generation. Such a “hypercoagulability state” is characterized by an increased risk of venous and arterial thrombosis, as well as predisposition to warfarin-associated microvascular thrombosis (eg, venous limb gangrene). However, it is also possible that other unique pathogenetic mechanisms operative in HIT explain unusual thromboses, such as arterial “white clots.” For example, HIT antibodies have been shown to activate endothelium and monocytes (leading to cell surface tissue factor expression), although this stimulation may be large indirect through poorly defined mechanisms involving platelet activation and, possibly, formation of platelet-derived microparticles. Further, aggregates of platelets and polymorphonuclear (PMN) leukocytes have been described in HIT. To what extent these cooperative interactions between platelets, microparticles, PMN leukocytes, monocytes, and endothelium lead to arterial or venous thrombotic events in HIT, either in large or small vessels, remains unclear. (Used with permission from Warkentin.<sup>164</sup>)



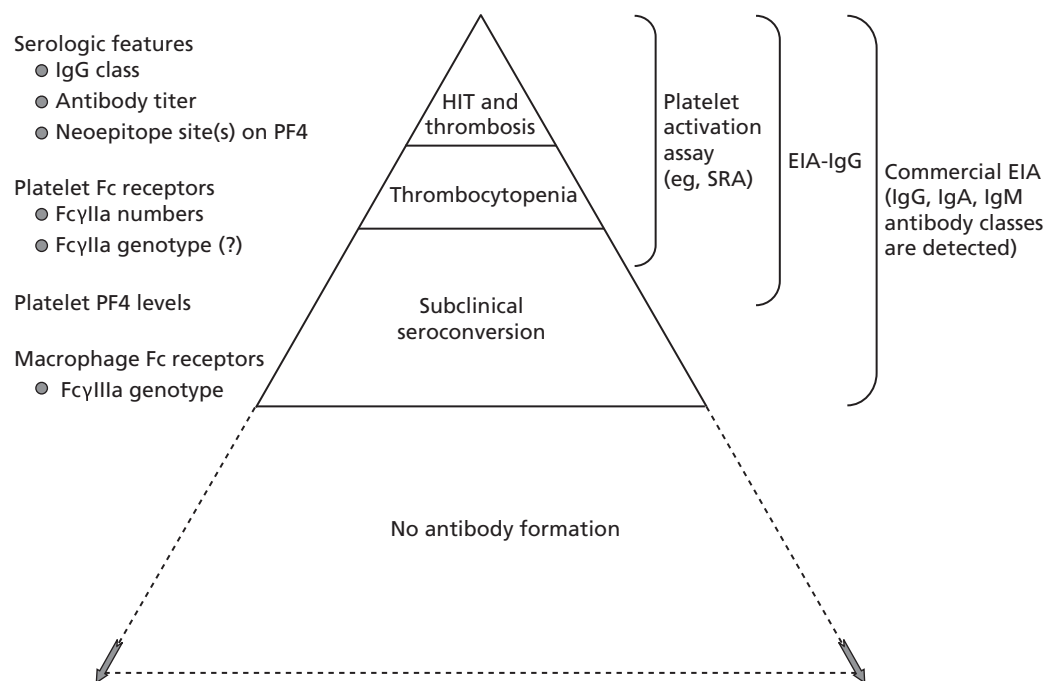


**Figure 23-5.** This “iceberg” model depicts several features of HIT, including the hierarchy of sensitivity and specificity of three different types of assays: 1) platelet activation assay that utilizes washed platelets, eg, platelet serotonin-release assay (SRA); 2) PF4/heparin enzyme immunoassay that detects IgG antibodies (EIA-IgG); and 3) commercial EIA that detects IgG, IgM, and/or IgA antibodies. Clinical HIT occurs in either of the top two levels of the iceberg (HIT and thrombosis;

thrombocytopenia). Subclinical seroconversion indicates formation of antibodies in the absence of developing clinical HIT. The relative proportion of patients who form antibodies vs those who do not form antibodies differs in various clinical situations. Various serologic, platelet, and macrophage characteristics that determine the pathogenicity of PF4/heparin-reactive antibodies are shown on the left. (Modified with permission from Warkentin, Cook.<sup>170</sup>)

activation assay or PF4-dependent EIA.<sup>170</sup> The key concept is that whereas both types of assay have high sensitivity for diagnosis of clinical HIT, the former assay has greater diagnostic specificity. It is estimated that substantial overdiagnosis (~100%) of HIT will result<sup>171</sup> if a positive EIA of any magnitude is considered diagnostic of HIT irrespective of the patient’s clinical likelihood of HIT, as judged using a clinical scoring system (the 4T’s)<sup>172</sup> and the gold standard assay (platelet SRA).

### Management of Heparin-Induced Thrombocytopenia-Associated Thrombosis

Results of prospective and retrospective studies<sup>2,3,162,172</sup> indicate that approximately 50% to 75% of patients with HIT have new, progressive, or recurrent thrombosis. Thus, the need for an alternative anticoagulant is a common issue in the care of these patients.

*Danaparoid sodium* (Orgaran, Schering-Plough, Kenilworth, NJ) is a mixture of anticoagulant glycosaminoglycans with predominant anti-Factor Xa activity that decreases thrombin generation in patients with HIT.<sup>173</sup> A randomized trial showed a higher thrombosis resolution rate among patients treated with danaparoid and warfarin than among those treated with dextran and warfarin, especially for patients with severe thrombosis (92% vs 33%;  $p < 0.001$ ).<sup>174</sup> Although some HIT sera show in-vitro

cross-reactivity with danaparoid (ie, enhanced platelet activation in the presence of danaparoid), this effect is usually weak; moreover, platelet-activating effects of many HIT sera are inhibited by danaparoid at therapeutic concentrations.<sup>174</sup> Because in-vitro cross-reactivity is not predictive of adverse outcome,<sup>174</sup> danaparoid should be given without preceding in-vitro testing for cross-reactivity.<sup>159</sup> The success rate is high (approximately 85% to 90%), as defined by platelet count recovery without new thrombosis.<sup>159,174,175</sup> The anticoagulant effect of danaparoid is monitored with a chromogenic anti-Factor Xa assay, which must be performed using a standard curve prepared with danaparoid. The target range is generally 0.5 to 0.8 anti-Factor Xa U/mL, although levels of 1.0 U/mL or higher are used by some in the treatment of patients with severe thrombosis. Recommended therapeutic dosing includes an initial intravenous bolus followed by continuous infusion (usually 200 U/hour with monitoring of anti-Factor Xa levels, if available). Danaparoid does not interfere with INR measurements. This is an advantage in the care of patients with venous thromboembolism in whom overlapping warfarin anticoagulation usually is performed after resolution of thrombocytopenia. Danaparoid is approved therapy for HIT in the European Union, Canada, and elsewhere, but is neither approved for HIT management nor currently available in the United States.

*Lepirudin* (Refludan, Bayer HealthCare, Leverkusen, Germany) is a hirudin derivative manufactured by means of recombinant technology. Two prospective cohort studies<sup>176,177</sup> of lepirudin with a prespecified dosing schedule for the management of HIT-associated thrombosis (with historical controls for comparison) led to approval of this agent in the United States, Canada, and the European Union for treatment of patients with HIT-associated thrombosis. Meta-analysis<sup>178</sup> of these two studies showed that a subtherapeutic activated partial thromboplastin time (aPTT) ratio ( $<1.5$ ) was associated with increased risk of thrombosis, whereas a therapeutic aPTT ratio above the therapeutic range ( $>2.5$ ) was associated with increased bleeding without further reduction in antithrombotic efficacy. However, even for patients within the target therapeutic range, the risk of bleeding was significantly greater than among historical controls (relative risk approximately 3). Current dosing recommendations<sup>159</sup> are considerably lower than that recommended by the manufacturer, in keeping with postapproval studies indicating that the approved dosing regimen is too high.<sup>179,180</sup>

The anticoagulant effect of lepirudin is usually monitored with the aPTT. Lepirudin is renally excreted, and ideally should not be used to treat patients with renal failure because there is substantial risk of overdosing, and no antidote exists. The half-life of lepirudin is short (approximately 1.3 hours) in patients with normal renal function. A high proportion of patients develop hirudin antibodies, which occasionally results in a paradoxical increase in the anticoagulant effects of the drug (perhaps via reduced renal clearance). Thus, ongoing aPTT monitoring is recommended during lepirudin treatment, even if initial anticoagulation is stable. Fatal anaphylactoid reactions have been reported following use of an intravenous bolus, particularly in patients sensitized because of recent exposure to hirudin.<sup>159</sup>

*Argatroban* (Novastan, GlaxoSmithKline, Middlesex, United Kingdom) is a small-molecule, direct thrombin inhibitor approved in the United States for the management and prevention of HIT-associated thrombosis.<sup>159,181</sup> Advantages of argatroban include its short half-life (40–50 minutes) as well as its hepatic route of metabolism. No dose adjustments are needed for patients with moderate renal failure, although dose reduction is needed in the presence of hepatic insufficiency. Anticoagulant monitoring is usually performed using the aPTT. Argatroban also prolongs the INR, which complicates the transition from argatroban to warfarin therapy. It is important to postpone introduction of warfarin until the thrombocytopenia has substantially recovered (platelet count at least 150,000/ $\mu$ L).<sup>159</sup>

There are several important contraindications to therapy for acute HIT, including warfarin monotherapy, LMW heparin, and prophylactic platelet transfusion.<sup>159</sup> Warfarin therapy can lead to acute depletion of protein C, which can cause microvascular thrombosis in HIT, and lead to venous limb gangrene.<sup>165</sup> However, once thrombocytopenia has resolved, it is reasonable to overlap warfarin cautiously with one of the agents that can reduce thrombin generation in HIT (danaparoid, lepirudin, argatroban). Use of LMW heparin is contraindicated because of a

high chance of treatment failure (approximately 50% of patients have further thrombocytopenia or thrombosis).<sup>159</sup> Prophylactic platelet transfusion is relatively contraindicated because even patients with severe thrombocytopenia do not usually have evidence of hemostatic dysfunction, such as petechiae, and platelet transfusions may contribute to increase risk of thrombosis.<sup>159</sup>

### Management of Isolated Heparin-Induced Thrombocytopenia

Isolated HIT is defined as HIT that is recognized because of thrombocytopenia without evidence of HIT-associated thrombosis.<sup>162</sup> Unfortunately, simply stopping administration of heparin or substituting warfarin for heparin is inadequate treatment for these patients. In a large retrospective cohort study,<sup>162</sup> the risk of thrombosis among these patients was approximately 10% at 2 days, 40% at 7 days, and 53% at 30-day follow-up evaluations. Other investigators<sup>182</sup> with a similar approach subsequently found a 38% rate of thrombotic events despite stopping administration of heparin. The frequency of thrombosis surprisingly was not any lower in the subgroup of patients for whom heparin was stopped fairly promptly ( $<48$  hours) after the onset of thrombocytopenia than it was among patients with later cessation of heparin (45 vs 34%;  $p = 0.26$ ).

For patients strongly suspected (or confirmed) to have isolated HIT, the authors discontinue heparin, start administration of an alternative rapidly acting anticoagulant, and screen for subclinical DVT by means of compression ultrasonography (approximately 50% of patients are shown to have DVT with this approach).<sup>159</sup> Whether or not thrombosis is found, a therapeutic dose of anticoagulant is given to these patients. This is because prophylactic-dose anticoagulation appears to have a higher failure rate than does therapeutic-dose anticoagulation.<sup>175</sup> Further, argatroban was approved in therapeutic doses for prevention of thrombosis in the care of patients with HIT.<sup>181</sup> After platelet count recovery, the absence of venous thrombosis is confirmed before discharge. For patients found at initial or follow-up imaging to have venous thrombosis, overlapping warfarin anticoagulation is usually begun for longer-term antithrombotic control.

### Reexposure to Heparin in a Patient with a History of Heparin-Induced Thrombocytopenia

Patients who have circulating HIT antibodies can have an abrupt decrease in platelet count if heparin is administered. However, the risk of such abrupt-onset HIT on reexposure to heparin is restricted to the first few months after use of heparin. This is because HIT antibodies begin to decline after heparin is discontinued, and usually they are no longer detectable by the 3-month follow-up evaluation.<sup>183</sup> Under exceptional circumstances (eg, need to perform heart or vascular surgery), it is recommended to readminister heparin to a patient with previous HIT, provided that HIT antibodies are no longer detectable with a sensitive and reliable assay (eg, platelet SRA or PF4-dependent EIA).<sup>159,183</sup> Such patients often do not form HIT antibodies after the brief reexposure to heparin, and if they do, these antibodies are not



formed before Day 5. Nevertheless, it seems prudent to limit the heparin reexposure to the operation itself and to use an alternative anticoagulant for perioperative anticoagulation.

### Typical Drug-Induced Immune Thrombocytopenia

The clinical criteria supporting a diagnosis of DITP are 1) thrombocytopenia occurs during drug treatment and is corrected completely after discontinuation of the drug; 2) the implicated drug was the only one used when thrombocytopenia occurred, or platelet count recovery occurred or persisted despite continuation or reintroduction of the other drugs used; 3) other causes of thrombocytopenia are excluded; and 4) reexposure to the implicated agent resulted in recurrent thrombocytopenia.<sup>154</sup> For reasons of patient safety, drug reexposure is rarely performed deliberately, but sometimes the outcome of unintentional reexposure can provide important diagnostic information (eg, recurrent thrombocytopenia following ingestion of tonic water suggests quinine-induced thrombocytopenia). Meeting all four criteria provides definite evidence of causation, whereas meeting the first three criteria suggests a probable cause.<sup>154</sup>

Drug-induced immune-mediated thrombocytopenia typically begins abruptly and is severe, most patients having a platelet count less than 20,000/ $\mu$ L.<sup>4,156,160</sup> Although the interval between starting the drug and development of thrombocytopenia usually is 1 or 2 weeks, occasionally it can be several months or even longer after administration of the drug is started. Sometimes, thrombocytopenia persists for several weeks even after the drug is stopped, possibly because some of the IgG antibodies formed have drug-independent platelet reactivity.

Although many dozens of drugs have been suspected as causing DITP, convincing evidence to support a causal relation exists for relatively few drugs.<sup>153-156</sup> Supporting evidence for the drugs claimed to cause DITP is available (<http://moon.ouhsc.edu/jgeorge>). The risk of DITP is approximately 1 case in 1000 for quinine, and 1 in 25,000 for sulfamethoxazole-trimethoprim.<sup>184</sup> Quinine is present in certain beverages (eg, tonic water) and consequently patients may not be aware of exposure.

The pathogenesis of DITP involves formation of a ternary complex involving a platelet glycoprotein (usually, the GPIIb/IIIa complex, less often GPIb/IX), drug (or drug metabolite), and the Fab terminus of IgG.<sup>7,151,185</sup> Such a mechanism has been invoked for quinine, quinidine, sulfonamide, rifampin, vancomycin,<sup>151</sup> and pentamidine, among others. Unlike the mechanism of HIT, platelet Fc $\gamma$ Rs are not involved. Further, the drug does not function as a hapten; that is, drug-dependent IgG does not bind to platelets that have been washed after pretreatment with the implicated drug.<sup>156</sup> Limited evidence of a hapten mechanism of DITP has been suggested only for penicillin; that is, penicillin-dependent IgG binds to platelets that have been washed after pretreatment with penicillin. A recently proposed model suggests that drug-dependent antibodies are derived from a pool of naturally occurring antibodies with weak affinity for self antigens residing on platelet membrane glycoproteins; certain drugs are able to affect both antibody and antigen in such a way that their

interaction is greatly enhanced, provided that B cells expressing such antibodies are induced to produce these.<sup>156,186</sup>

In a case of suspected DITP, as many drugs as possible should be discontinued. If further drug treatment is necessary, an immunologically non-cross-reactive substitute should be prescribed. Platelet transfusions should be given to patients with life-threatening bleeding or who are judged to be at high risk of bleeding (eg, severe thrombocytopenia plus “wet purpura”). High-dose IVIG, 1 g/kg given over 6 to 8 hours for 2 consecutive days, may be of value, but can be ineffective if the relevant drug is not discontinued.<sup>151</sup> Corticosteroids are relatively ineffective in the management of this condition.<sup>160</sup>

### Atypical Drug-Induced Immune Thrombocytopenic Purpura

#### Glycoprotein IIb/IIIa Antagonist-Induced Thrombocytopenia

Thrombocytopenia is a relatively common side effect of the three approved GPIIb/IIIa antagonists—abciximab, tirofiban, and eptifibatide.<sup>161</sup> Often the thrombocytopenia begins within hours of a first exposure; this is caused by naturally occurring antibodies.<sup>187</sup> In other patients, the thrombocytopenia begins about 1 week after initial—or abruptly upon repeat—drug administration; this clinical presentation reflects drug-induced antibody formation.<sup>188</sup>

Abciximab (c7E3, ReoPro, Eli Lilly, Indianapolis, IN) is a humanized chimeric Fab fragment of a murine monoclonal antibody specific for an epitope on GPIIIa. It is used to prevent restenosis after coronary angioplasty. Approximately 0.5% of patients have moderate or severe thrombocytopenia within several hours of treatment with this drug.<sup>161</sup> Although approximately 20% of the normal population have antibodies that react against the papain cleavage site in abciximab, the remainder (1.6% overall) react against murine sequences incorporated into abciximab that confer specificity for GPIIb/IIIa. It is this latter group of antibodies that evinces pathogenicity. Perhaps surprisingly, given the degree of thrombocytopenia and use of a major platelet glycoprotein inhibitor, most patients do not have petechiae or bleeding<sup>161</sup> (although fatal bleeding episodes have been reported). Treatment with platelet transfusions and, perhaps, IVIG may benefit bleeding patients.

For approximately one-third of patients with apparent abciximab-associated thrombocytopenia, examination of the blood film shows platelet clumping. This finding suggests pseudothrombocytopenia (ex-vivo platelet clumping) caused by abciximab.<sup>189</sup> Such patients are not at risk for bleeding and do not need treatment.

Tirofiban and eptifibatide are synthetic compounds that mimic or contain the RGD (arg-gly-aspartic acid) peptide and bind tightly to the RGD recognition site in GPIIb/IIIa.<sup>161</sup> As with abciximab, the frequency of naturally occurring antibodies (approximately 1% to 2%) correlates roughly with their risk of inducing abrupt-onset thrombocytopenia upon first exposure. Delayed-onset thrombocytopenia beginning about 1 week after exposure has also been reported.

### Drug-Induced Autoimmune Thrombocytopenia

Approximately 1% to 3% of patients treated with gold have thrombocytopenia that sometimes persists for weeks or months despite stopping the drug; thus the disorder resembles chronic ITP.<sup>190</sup> It remains uncertain whether this is true drug-induced autoimmune thrombocytopenia or is caused by gold-dependent IgG antibodies that are slowly released from tissues. Procainamide,  $\alpha$ -methyl dopa, sulphonamide antibiotics, and interferons alfa and beta also may cause autoimmune thrombocytopenia.<sup>156,191</sup> In extremely rare instances, measles-mumps-rubella vaccination causes an acute ITP-like illness in which anti-GPIIb/IIIa IgG is formed.<sup>192</sup>

### Drug-Induced Thrombotic Thrombocytopenic Purpura and Hemolytic Uremic Syndrome

In rare instances, drugs cause a thrombotic microangiopathy that closely resembles TTP or HUS. Paradoxically, such platelet-mediated thrombotic disorders can even be caused by the antiplatelet agents ticlopidine<sup>193</sup> and clopidogrel.<sup>194</sup> Ticlopidine-induced TTP is estimated to occur in 1 in 2000 to 5000 patients who receive this drug after coronary stenting. The characteristic onset is between 1 and 8 weeks after administration of the drug is started. Clopidogrel-induced TTP occurs in approximately 1 in 20,000 recipients, generally within the first 2 weeks of treatment. Autoantibodies to von Willebrand factor-cleaving metalloproteinase have been identified in these patients and may contribute to the pathogenesis.<sup>195</sup>

Other drugs that cause an illness that resembles HUS include quinine,<sup>196</sup> antineoplastic chemotherapy (eg, gemcitabine, mitomycin C, cisplatin, bleomycin), immunosuppressive agents [eg, cyclosporin, FK506 (tacrolimus)], and miscellaneous drugs (penicillamine, penicillin). Because some of the medical conditions leading to use of these drugs can be associated with microangiopathic blood film changes (neoplasia, organ rejection, graft-vs-host disease, vasculitis), causal relationships to the various drugs listed can be problematic.

The mortality of drug-induced TTP is high. Early recognition and discontinuation of the drug are essential. Response to plasma exchange has been observed. Many physicians would also give corticosteroids, although the efficacy remains unproven. Specific therapy for drug-induced HUS (dialysis, plasmapheresis, corticosteroids) is individualized depending on the clinical situation. The prognosis is poor for drug-induced TTP in the setting of HSCT.

## Alloimmune Thrombocytopenia

Alloantigens are genetically determined molecular variations of proteins or carbohydrates that can be recognized immunologically by some healthy persons. Exposure to alloantigens occurs during pregnancy, transfusion, or transplantation. If alloantibodies form against platelet alloantigens, alloimmune thrombocytopenia can result from platelet clearance mediated by the reticuloendothelial system. Five alloimmune thrombocytopenic disorders have been described (Table 23-3),<sup>197</sup> the most common being NAIT.

**Table 23-3.** The Five Alloimmune Thrombocytopenic Syndromes<sup>197</sup>

#### Classical alloimmune thrombocytopenic syndromes

- Neonatal alloimmune thrombocytopenia
- Posttransfusion purpura

#### Other alloimmune thrombocytopenic syndromes

- Passive alloimmune thrombocytopenia
- Transplantation-associated alloimmune thrombocytopenia
- Platelet transfusion refractoriness

### Alloantigens

More than 20 platelet alloantigens have been identified.<sup>197-203</sup> Table 23-4 classifies the platelet alloantigens by glycoprotein localization and gene frequency, the latter divided into public and private (or low frequency, arbitrarily less than 0.02). More than one-half of the alloantigens that have been identified are located on one of the two glycoproteins that constitute the GPIIb/IIIa complex (platelet fibrinogen receptor). One of these alloantigens, HPA-1a (PI<sup>A1</sup>), is located on GPIIIa. It is responsible for most alloimmune thrombocytopenia in populations of European ancestry, including almost all patients with severe alloimmune thrombocytopenia. The other major platelet glycoprotein complex (GPIb/IX, von Willebrand factor-binding complex) is rarely implicated in alloimmune thrombocytopenia. However, the GPIa/IIa complex (platelet collagen receptor), which bears the HPA-5a/5b (Br<sup>a/b</sup>; Za<sup>a/b</sup>) alloantigen system, is a relatively common cause of moderately severe alloimmune thrombocytopenia.<sup>204</sup> The HPA-15a/15b (Gov<sup>a/b</sup>) alloantigen system has been shown to be a relatively common cause of alloimmune thrombocytopenia that, like HPA-5a/5b, tends not to be severe.<sup>205</sup>

### Immunogenetics and Frequency of Alloimmune Thrombocytopenia

The HPA-1a alloantigen is far more immunogenic than its corresponding allele, HPA-1b. For example, consider the frequency of NAIT caused by either anti-HPA-1a or anti-HPA-1b alloantibodies in relation to the genotype frequency of HPA-1a (0.85) and HPA-1b (0.15). Thus, a homozygous HPA-1bb (PI<sup>A1</sup>-negative) female, representing approximately 2% ( $0.15 \times 0.15$ ) of the population, would have an 85% probability of being exposed to the HPA-1a alloantigen during pregnancy. In contrast, a homozygous HPA-1aa female, representing approximately 72% ( $0.85 \times 0.85$ ) of the population, would have a 15% probability of being exposed to the HPA-1b alloantigen during pregnancy. Thus, if both alloantigens were equally immunogenic, one would expect anti-HPA-1b to occur approximately six times more often than anti-HPA-1a:  $(0.72 \times 0.15)/(0.02 \times 0.85) = 6.4$ . However, the opposite is actually observed: anti-HPA-1a is far more common than anti-HPA-1b (Table 23-5).<sup>204,206</sup> In a study of 348 cases of suspected NAIT,<sup>204</sup> only one case caused by anti-HPA-1b was found, compared with 144 cases of proven or suspected NAIT secondary to anti-HPA-1a. Thus, the observed ratio of NAIT caused by anti-HPA-1a/anti-HPA-1b (1:144, or

**Table 23-4.** Platelet Antigens Classified According to Glycoprotein Location and Gene Frequency\*

| Platelet-Specific Alloantigen<br>(Alternative Nomenclature)         | GP          | Gene Frequency in Whites | NAIT | PTP | PAT | TAT | PTR |
|---------------------------------------------------------------------|-------------|--------------------------|------|-----|-----|-----|-----|
| <b>GPIIb/IIIa: public</b><br>(gene frequency > 0.02)                |             |                          |      |     |     |     |     |
| HPA-1a (Pl <sup>A1</sup> , Zw <sup>a</sup> )                        | IIla        | 0.85                     | ++   | ++  | +   | +   | (+) |
| HPA-1b (Pl <sup>A2</sup> , Zw <sup>b</sup> )                        | IIla        | 0.15                     | +    | +   | —   | —   | (+) |
| HPA-3a (Bak <sup>a</sup> , Lek <sup>a</sup> )                       | IIb         | 0.61                     | +    | +   | —   | —   | (+) |
| HPA-3b (Bak <sup>b</sup> )                                          | IIb         | 0.39                     | ?    | +   | —   | —   | (+) |
| HPA-4a (Pen <sup>a</sup> , Yuk <sup>b</sup> )                       | IIla        | >0.99                    | +    | +   | —   | —   | (+) |
| <b>GPIIb/IIIa: private/low-frequency</b><br>(gene frequency < 0.02) |             |                          |      |     |     |     |     |
| HPA-4b (Pen <sup>b</sup> , Yuk <sup>a</sup> )                       | IIla        | <0.01                    | +    | —   | —   | —   | —   |
| HPA-6b (Tu <sup>a</sup> , Ca <sup>a</sup> )                         | IIla        | 0.003                    | +    | —   | —   | —   | —   |
| HPA-7b (Mo <sup>a</sup> )                                           | IIla        | <0.01                    | +    | —   | —   | —   | —   |
| HPA-8b (Sr <sup>a</sup> )                                           | IIla        | <0.003                   | +    | —   | —   | —   | —   |
| HPA-9b (Max <sup>a</sup> )                                          | IIb         | 0.002                    | +    | —   | —   | —   | —   |
| HPA-10b (La <sup>a</sup> )                                          | IIla        | <0.01                    | +    | —   | —   | —   | —   |
| HPA-11b (Gro <sup>a</sup> )                                         | IIla        | <0.001                   | +    | —   | —   | —   | —   |
| HPA-14b (Oe <sup>a</sup> )                                          | IIla        | <0.005                   | +    | —   | —   | —   | —   |
| HPA-16b (Duv <sup>a</sup> )                                         | IIla        | <0.01                    | +    | —   | —   | —   | —   |
| Va <sup>a</sup>                                                     | IIb/IIla    | <0.002                   | +    | —   | —   | —   | —   |
| <b>GP Ia/IIa: public</b>                                            |             |                          |      |     |     |     |     |
| HPA-5a (Br <sup>b</sup> , Zav <sup>b</sup> )                        | Ia          | 0.89                     | +    | ?   | —   | —   | (+) |
| HPA-5b (Br <sup>a</sup> , Zav <sup>a</sup> , Hc <sup>a</sup> )      | Ia          | 0.11                     | ++   | +   | +   | +   | (+) |
| <b>GP Ia/IIa: private</b>                                           |             |                          |      |     |     |     |     |
| HPA-13b (Sit <sup>a</sup> )                                         | Ia          | 0.0025                   | +    | —   | —   | —   | —   |
| Swi <sup>a</sup>                                                    | Ia          | <0.002                   | +    | —   | —   | —   | —   |
| <b>GP Ib/IX: public</b>                                             |             |                          |      |     |     |     |     |
| HPA-2a (Ko <sup>b</sup> )                                           | Ib $\alpha$ | 0.89                     | —    | —   | —   | —   | ?   |
| HPA 2b (Ko <sup>a</sup> , Sib <sup>a</sup> )                        | Ib $\alpha$ | 0.11                     | +    | ?   | —   | —   | (+) |
| <b>GP Ib/IX: private</b>                                            |             |                          |      |     |     |     |     |
| HPA-12b (Iy <sup>a</sup> )                                          | Ib $\beta$  | <0.01                    | +    | —   | —   | —   | —   |
| <b>CD109: public</b>                                                |             |                          |      |     |     |     |     |
| HPA-15a (Gov <sup>b</sup> )                                         | CD109       | 0.53                     | +    | ?   | —   | —   | —   |
| HPA-15b (Gov <sup>a</sup> )                                         | CD109       | 0.47                     | +    | —   | —   | —   | (+) |
| <b>GP38</b>                                                         |             |                          |      |     |     |     |     |
| Dy <sup>a</sup>                                                     | 38 kD       | <0.01                    | +    | —   | —   | —   | —   |
| <b>Platelet nonspecific<br/>alloantigens</b>                        |             |                          |      |     |     |     |     |
| ABO                                                                 |             |                          | —    | —   | —   | —   | ++  |
| HLA                                                                 |             |                          | —    | —   | —   | —   | ++  |

\*Modified with permission from Warkentin & Smith.<sup>197</sup>

GP = glycoprotein; NAIT = neonatal alloimmune thrombocytopenia; PTP = posttransfusion purpura; PAT = passive alloimmune thrombocytopenia; TAT = transplantation-associated alloimmune thrombocytopenia; PTR = platelet transfusion refractoriness.

++ = relatively common; + = established but rare; — = not reported; (+) = probable association, but definitive link inconclusive; ? = possible association but not established.

0.007) is almost 1000 times less than predicted with the theoretical ratio (6.4).

Immunogenetics is a major factor determining alloimmunization against HPA-1a. There is a strong association

between formation of anti-HPA-1a and *HLA-DRB3\*0101* and *HLA-DQB1\*0201* (odds ratio, 25 and 40, respectively).<sup>207</sup> In contrast, no HLA association exists for immunization against HPA-1b.<sup>208</sup> Thus, it appears that persons with certain HLA genotypes

**Table 23-5.** Observed Frequencies of Neonatal Alloimmune Thrombocytopenia and Posttransfusion Purpura in Relation to Expected (Theoretic) Frequency of the HPA-1ab (Pl<sup>A1/A2</sup>), HPA-5ab (Br<sup>a/b</sup>), and HPA-3ab (Ba<sup>a/b</sup>) Alloantigen Systems\*

| Target Alloantigen | Percentage of Pregnancies at Theoretic Risk of NAIT <sup>†</sup> (Descending Order) | Observed Cases of NAIT <sup>‡</sup> | Observed Cases of PTP <sup>§</sup> |
|--------------------|-------------------------------------------------------------------------------------|-------------------------------------|------------------------------------|
| HPA-3b             | 14.5                                                                                | 0                                   | 0                                  |
| HPA-1b             | 10.8                                                                                | 0                                   | 29                                 |
| HPA-3a             | 9.3                                                                                 | 1                                   | 9                                  |
| HPA-5b             | 8.7                                                                                 | 6                                   | 11                                 |
| HPA-1a             | 1.9                                                                                 | 44                                  | 105                                |
| HPA-5a             | 1.1                                                                                 | 0                                   | 7                                  |

\*Modified with permission from Warkentin & Smith.<sup>197</sup>

<sup>†</sup>Percentage of pregnancies at theoretic risk of NAIT for a given target alloantigen is determined as follows:  $x(1 - x)^2 \times 100$ , where  $x$  is the gene frequency of the target alloantigen. Note the lack of correlation between the theoretical and observed risk for NAIT.

<sup>‡</sup>Data are from Mueller-Eckhardt et al.<sup>204</sup> and represent serologic investigations using a defined protocol over an 18-month period ending June 30, 1988.

<sup>§</sup>For comparison, the serologic findings for cases of PTP are shown for which only one platelet alloantigen specificity was identified (from January 1990 to August 2006 at the BloodCenter of Wisconsin).<sup>206</sup>

NAIT = neonatal alloimmune thrombocytopenia; PTP = posttransfusion purpura.

are much more likely to generate an alloimmune response when GPIIIa bears the leucine<sup>33</sup> substitution that determines the HPA-1a phenotype.

Overall, on the basis of the observed allelic frequencies, the expected theoretical ratio of NAIT for anti-HPA-5b, compared with anti-HPA-5a, should be approximately 8 (Table 23-5). A similar ratio (47:3, or 15.7) has been observed. However, although the expected and observed ratios are similar (contrast the HPA-1a/1b system), a role for immunogenetics and alloimmunization exists also for the HPA-5a/5b system.<sup>209</sup>

### Severity of Alloimmune Thrombocytopenia

In general, the severity of thrombocytopenia is greater for alloimmune thrombocytopenia that involves the GPIIb/IIIa complex, compared with the GPIa/IIa complex (Table 23-6<sup>197,204,206,210</sup>). Because there are approximately 20 times more GPIIb/IIIa molecules compared with GPIa/IIa complexes (40,000 vs 2000), this suggests that greater numbers of alloantibodies binding to the more numerous GPIIb/IIIa receptors results in greater platelet destruction.

### Neonatal Alloimmune Thrombocytopenia

Neonatal alloimmune thrombocytopenia is a transient but potentially life-threatening thrombocytopenic disorder limited to fetal and neonatal life. It is caused by maternal IgG alloantibodies that cross the placenta and cause premature destruction of platelets bearing paternally derived platelet alloantigens (analogous to

**Table 23-6.** Severity of Thrombocytopenia by Platelet Count Nadirs ( $\times 1000/\mu\text{L}$ ) in Relation to Target Glycoprotein for Various Alloimmune Thrombocytopenic Syndromes\*

| Glycoprotein      | NAIT        | PTP        | PAT        | TAT        |
|-------------------|-------------|------------|------------|------------|
| <b>GPIIb/IIIa</b> |             |            |            |            |
| HPA-1a (IIIa)     | 17 (n = 81) | 6 (n = 43) | 8 (n = 9)  | 8 (n = 4)  |
| HPA-1b (IIIa)     | 9 (n = 2)   | 5 (n = 4)  | —          | —          |
| HPA-3ab (IIb)     | 10 (n = 5)  | 3 (n = 4)  | —          | —          |
| HPA-4ab (IIIa)    | 13 (n = 7)  | 6 (n = 1)  | —          | —          |
| Mean              | 16          | 6          | 8          | 8          |
| <b>GPIa/IIa</b>   |             |            |            |            |
| HPA-5b            | 44 (n = 48) | 26 (n = 1) | 35 (n = 1) | 43 (n = 1) |
| HPA-5a            | 35 (n = 5)  | —          | —          | —          |
| Mean              | 43          | 26         | 35         | 43         |

\*Data from Warkentin & Smith<sup>197</sup> and Brunner-Bolliger et al.<sup>210</sup>

The data show that alloimmune thrombocytopenic syndromes that involve GPIIb/IIIa are more likely to cause severe thrombocytopenia than are those involving GPIa/IIa. The data are combined for alloimmune thrombocytopenic syndromes involving either allele of the HPA-3ab and HPA-4ab alloantigen systems, whereas the data are shown separately for the alleles of the HPA-1ab and HPA-5ab systems.

NAIT = neonatal alloimmune thrombocytopenia; PTP = posttransfusion purpura; PAT = passive alloimmune thrombocytopenia; TAT = transplantation-associated alloimmune thrombocytopenia.

hemolytic disease of the fetus and newborn). Neonatal alloimmune thrombocytopenia occurs in approximately 1 to 1.5 per 1000 live births.<sup>211</sup>

Approximately 75% of cases in a population of European ancestry are caused by fetomaternal incompatibility for the platelet-specific alloantigen HPA-1a, and 20% by HPA-5b.<sup>204</sup> Other alloantigens implicated in NAIT, including private alloantigens identified in only one or a few families [eg, HPA-6b (Tu<sup>a</sup>/Ca), HPA-7b (Mo<sup>a</sup>)], are shown in Table 23-4. In east Asian populations, anti-HPA-4b (Pen<sup>b</sup>) is more common than anti-HPA-1a. Although HLA or ABO alloantibodies have been claimed to cause NAIT, in most cases, undetected platelet-specific alloantibodies or another diagnosis caused the thrombocytopenia.<sup>212</sup>

The typical clinical presentation of NAIT is isolated severe thrombocytopenia in an otherwise healthy neonate, especially if fetomaternal incompatibility involves an alloantigen on the GPIIb/IIIa complex (Table 23-6). Petechiae are found in 90%; gastrointestinal tract hemorrhage in 30%; and hemoptysis, hematuria, and retinal bleeding in fewer than 10% of patients. Approximately 15% have intracranial hemorrhage.<sup>204</sup> The thrombocytopenia usually resolves within 1 to 3 weeks. Serious sequelae of fetal and neonatal intracranial bleeding include hydrocephalus, porencephalic cysts, and epilepsy. First-born offspring constitute approximately one-half of patients. This suggests that unlike the situation for hemolytic disease of the

fetus and newborn, sensitization can occur early during the first pregnancy.<sup>204</sup> Subsequently affected siblings usually have thrombocytopenia to a similar or greater extent, an observation used to emphasize preventive treatment in subsequent pregnancies.

Laboratory investigation of possible NAIT involves three steps. First, there must be a high index of suspicion: isolated thrombocytopenia in an otherwise well infant should be assumed to indicate NAIT until proved otherwise. The second step is to type maternal and paternal platelets to determine whether they are incompatible for a major platelet alloantigen. Commonly, the mother lacks certain platelet alloantigens that often are associated with alloimmune thrombocytopenia; for example, maternal homozygous HPA-1bb (PI<sup>A1</sup>-negative) status confers risk of NAIT caused by anti-HPA-1a. The third step is to determine whether the mother has platelet alloantibodies in her serum. Sometimes, no alloantibodies can be detected in maternal serum despite severe neonatal thrombocytopenia. Indeed, for approximately one-fourth of HPA-1bb mothers with infants believed to have had NAIT, anti-HPA-1a cannot be detected.<sup>204</sup> The potential for low-incidence platelet-specific alloantigens to explain fetomaternal incompatibility means that maternal serum should be tested against paternal platelets whenever possible. There remains debate as to whether the titer of alloantibodies predicts severity of fetal thrombocytopenia.

### Neonatal Treatment

The optimal treatment of a neonate in whom NAIT is suspected because of severe thrombocytopenia is to increase the platelet count urgently to safe levels, even before serologic confirmation of the diagnosis. In some centers (eg, National Blood Service in the United Kingdom), HPA-1bb and HPA-5bb platelets can be obtained upon request. These should be effective for more than 95% of patients.<sup>213</sup> When matched platelets are not available, washed and irradiated maternal platelets should be given to the neonate. These platelets are obtained by apheresis, and are washed to remove the maternal alloantibodies. Irradiation is performed to prevent graft-vs-host disease caused by maternal lymphocytes. In an emergency, immediate administration of whole-blood-derived platelets obtained from random donors may be of benefit to a bleeding infant.<sup>214</sup> Giving high-dose IVIG to the neonate increases the platelet count of approximately 65% of patients.<sup>215</sup> This treatment should be combined with maternally derived platelets. Corticosteroids are not recommended.

### Prenatal Management

About one-half of the time, NAIT is suspected during the prenatal period, usually because the mother previously bore an affected infant, although the diagnosis sometimes is suggested in utero when fetal ultrasonography shows cerebral hemorrhage, hydrocephalus, or hydrops fetalis. One tenet of management is that thrombocytopenia in a subsequently affected offspring is generally as severe as, or more severe than, a previously affected sibling. Neonatal alloimmune thrombocytopenia caused by anti-HPA-1a is more likely to cause fetal morbidity and mortality than

that caused by anti-HPA-5b and usually requires more aggressive treatment. When the father is known to be heterozygous for the implicated alloantigen (a situation that occurs approximately 25% and 20% of the time for NAIT involving the HPA-1a/1b and HPA-5a/5b systems, respectively), prenatal fetal typing (usually performed with genetic methods) is important, because it identifies the infant who is homozygous for the maternal antigen and is not at risk, obviating further treatment. For pregnancies at risk, general advice to the mother includes avoiding aspirin and nonsteroidal antiinflammatory medications.

Two general approaches have been taken to manage pregnancies at high risk of severe NAIT<sup>213,216</sup>: regular administration of high-dose IVIG, repeated in-utero platelet transfusions, or both. The initial step is to obtain a fetal platelet count by means of percutaneous umbilical blood sampling, generally starting at 20 to 24 weeks of gestation. Because of the risk of fetal exsanguination, maternal platelets should be on hand for transfusion if the fetal platelet count is shown to be less than 50,000/ $\mu$ L.<sup>216</sup> Intravenous immune globulin is given at a dosage of 1 g/kg/week starting within 1 week of documentation of fetal thrombocytopenia. Fetal blood sampling is repeated 4 to 6 weeks later; if no response is seen, glucocorticoid salvage treatment (prednisone, 60 mg/day) is started.<sup>216</sup> However, not all fetuses respond to this approach.

Another approach, which has been used in certain European centers, involves regular intrauterine platelet transfusions by means of percutaneous umbilical blood sampling, including a short time before delivery. This approach has led to good outcomes in situations in which previous siblings were severely affected.<sup>217</sup> However, each fetal platelet transfusion carries risk of hemorrhage and death<sup>218</sup> that likely depends on the experience of the fetomaternal unit. There is no consensus on which approach is preferred.

Regardless of the antenatal management, there is consensus that delivery should be by means of elective cesarean section, performed as soon as fetal maturity is documented. The major reason for this mode of delivery is that it allows an organized, multidisciplinary approach to the peripartum care of the newborn. This approach includes urgent determination of the cord platelet count, provision of washed, irradiated maternal platelets (or antigen-negative platelets) and, usually, the use of high-dose IVIG (1 g/kg/day for 2 consecutive days) to treat severe neonatal thrombocytopenia.

### Posttransfusion Purpura

Posttransfusion purpura is a very rare disorder that typically manifests as severe thrombocytopenia and bleeding that begin 5 to 10 days after blood transfusion—usually Red Blood Cells (RBCs)—in a patient previously sensitized by pregnancy or transfusion.<sup>206</sup> In 85% to 95% of cases, women are affected; the median age is 52 years. The observation that previous blood transfusions can be sensitizing explains why, on occasion, males develop PTP. Sometimes the presumably sensitizing transfusion occurs only a few weeks earlier; consequently, PTP can present after just a few weeks of intermittent transfusions.<sup>206</sup>

Although thrombocytopenia usually lasts 1 to 4 weeks, the duration can be as short as 3 days<sup>219</sup> to as long as 4 months

or more. The platelet count usually is less than 10,000/ $\mu$ L (Table 23-6). Mucocutaneous bleeding (wet purpura, petechiae, epistaxis, gastrointestinal, urinary tract) is common, and approximately 5% to 10% of patients die, usually because of intracranial hemorrhage. Because effective treatments are available (see below), it is important to diagnose PTP promptly to minimize morbidity and mortality. Diagnostic confusion with HIT can result because both syndromes can present 5 to 10 days following surgery, and sometimes PF4/heparin antibodies are present because of concomitant exposure to heparin.<sup>220,221</sup>

### Pathogenesis

Almost invariably, high-titer, platelet-specific alloantibodies are found in the patient's serum or plasma. Anti-HPA-1a is detected in 60% of cases, although several other platelet alloantigens have been implicated [HPA-1b, -2b, -3a, -3b, -4b, -5a, -5b, -15b, and the isoantigen CD-36 (Nak<sup>a</sup>)].<sup>206</sup> More than one specificity is observed in approximately 15% of cases. As in NAIT, the HLA-DRB3\*0101 antigen is found in most HPA-1a-negative patients with PTP.

Although platelet-specific alloantibodies are causative, the pathogenesis of PTP remains obscure, and the conundrum is why autologous platelets are destroyed. The currently favored hypothesis is that PTP represents a situation in which alloantibodies resulting from reexposure to an incompatible platelet alloantigen evince autospecificity ("pseudospecificity"). Although the platelet-specific alloantibodies are detectable for years following an episode of PTP, the autoreactive (or panreactive) antibodies are detectable only during the acute (thrombocytopenic) phase of PTP. In keeping with this view, Taaning and Tønnesen<sup>222</sup> reported that panreactive GPIIb/IIIa antibodies are readily detected during, but not after, an episode of PTP. Kiefel et al<sup>223</sup> reported that antibody with allospecificity for HPA-1a, but not for HPA-1b, could be eluted from both autologous and donor HPA-1bb platelets that had been sensitized with acute phase serum from a PTP patient, suggesting that use of adsorption/elution methods may help distinguish a reactivity profile of PTP sera from that seen with NAIT. One report<sup>224</sup> suggests that such alloantibodies with autoreactivity could arise spontaneously, because a woman with HPA-1bb platelets and no history of blood transfusion developed "ITP" with antibodies showing specificity for HPA-1a. Cure of the thrombocytopenia by splenectomy was accompanied by disappearance of the HPA-1a-like antibodies. Studies by another group of investigators<sup>225</sup> indicate that two distinct types of antibodies—some with alloreactivity and others with autoreactivity—develop during the acute phase of PTP.

### Treatment

High-dose IVIG is the treatment of choice for PTP. More than 90% of patients respond, attaining a platelet count greater than 100,000/ $\mu$ L in an average of 4 days.<sup>226</sup> Although some physicians also give corticosteroids, this agent probably does not influence the course of disease and should be considered adjunctive rather than primary therapy. In rare instances, splenectomy may be

considered for a patient refractory to IVIG, corticosteroids, and compatible platelet transfusion.<sup>227</sup>

Whole-blood-derived (unselected) platelets—which are likely to bear the HPA-1a antigen—are usually destroyed quickly, and can cause febrile or even anaphylactoid reactions. Antigen-negative platelets are the preferred component; however, the efficacy of HPA-1a-negative platelet transfusions (for patients with PTP caused by anti-HPA-1a) is also uncertain. Some reports indicate lack of benefit.<sup>228</sup> RBC units should be washed<sup>229</sup> or filtered<sup>228</sup> before administration to remove platelet antigens. Only four patients are known to have developed recurrent PTP with subsequent transfusions.<sup>206</sup> Accordingly, for a patient who has recovered from PTP, future precautions usually include avoidance of incompatible blood components (only autologous, washed, or platelet alloantigen-compatible RBCs are given, or platelet alloantigen-compatible plasma or platelet products are given). However, PTP recurrence is uncommon even if incompatible blood is given, possibly because residual high-titer platelet alloantibodies immediately clear the alloantigens. Patients with a history of PTP must not donate blood because their plasma can trigger passive alloimmune thrombocytopenia.

### Passive Alloimmune Thrombocytopenia

Passive alloimmune thrombocytopenia is characterized by abrupt onset of thrombocytopenia within a few hours after transfusion of a blood component.<sup>197</sup> It is caused by the passive transfer of platelet-reactive alloantibodies in the component that rapidly clear the incompatible recipient platelets. Protein-specific platelet-antibody studies confirm that the alloantibodies bind to the recipient's platelets. However, although the alloantibody can be detected in the donor's plasma, it may not be detectable in the recipient's plasma. This finding suggests that almost 100% of the transfused alloantibody binds soon after transfusion.<sup>210,230</sup> Only two alloantigens (HPA-1a and -5b) have been implicated in this syndrome.<sup>197,210,230</sup> In general, the severity of bleeding parallels the degree of thrombocytopenia; thus, spontaneous mucocutaneous bleeding usually occurs only in patients with severe thrombocytopenia caused by anti-HPA-1a. The duration of thrombocytopenia is generally less than 1 week. It is important to investigate suspected passive alloimmune thrombocytopenia, because the risk that numerous recipients can develop this syndrome means that the implicated blood donor must not donate blood in the future.

### Transplantation-Associated Alloimmune Thrombocytopenia

In rare instances, alloimmune mechanisms explain thrombocytopenia that occurs in the setting of HSCT or transplantation of solid organs.

### Hematopoietic Transplantation

Panzer et al<sup>231</sup> reported a 32-year-old man with chronic myeloid leukemia who had severe thrombocytopenia (platelet count, 17,000/ $\mu$ L) beginning 18 months after allogeneic marrow transplantation from his HLA-matched sister. High-dose IVIG gave

transient increases in platelet count, and persisting remission followed splenectomy. Antibodies with HPA-1a specificity were eluted from the patient's platelets. This led to further investigations, which showed that a small number of residual, non-neoplastic lymphoid cells of host origin produced anti-HPA-1a against the HPA-1a-positive platelets formed by donor-derived megakaryocytes. Thus, host-vs-donor alloimmune thrombocytopenia resulted from mixed chimerism, in which residual host lymphoid cells derived from the HPA-1a-negative individual developed an alloimmune response against platelets derived from the engrafted HPA-1a-positive marrow.

A similar situation attributable to anti-HPA-5b after allogeneic marrow transplantation for chronic myeloid leukemia has been reported.<sup>232</sup> However, in this patient, HPA-5b alloantibodies were detectable both before and after transplantation, and the early posttransplantation thrombocytopenia gradually improved as elutable anti-HPA-5b became more difficult to detect.

Alloimmune thrombocytopenia may have played a role in two cases with transfusion-refractory thrombocytopenia associated with a rise in titer of anti-HPA-1a (compared with the pretransplantation state) that developed following autologous HSCT performed for metastatic carcinoma of the breast. However, these reports are not conclusive, because it is difficult to distinguish a PTP-like illness (which implies destruction of engrafted autologous donor marrow-derived platelets) from typical posttransplantation platelet transfusion refractoriness.<sup>233</sup>

### Solid-Organ Transplantation

In rare instances, immunocompetent lymphoid cells within a transplanted solid organ cause alloimmune thrombocytopenia in the recipient of the organ. A dramatic scenario was reported by West et al.<sup>234</sup> All three organ recipients (two of a kidney, one of a liver) had severe thrombocytopenia and bleeding within 5 to 8 days after transplantation from a multiparous female organ donor with normal platelet counts. The two recipients of renal transplants had thrombocytopenia refractory to high-dose IVIG and platelet transfusions. One of these patients died, but the other recovered after splenectomy performed 50 days after transplantation. The liver transplant recipient had organ rejection, which was accompanied by correction of the platelet count when he received a new liver allograft. HPA-1a alloantibodies were detected in the organ donor and posttransplant (but not pretransplant) recipient serum. These cases illustrate that passenger immunocompetent lymphoid cells occasionally induce severe alloimmune thrombocytopenia when introduced into an alloincompatible environment.

### Platelet Transfusion Refractoriness

Platelet transfusion refractoriness, which is failure to achieve the expected platelet increment after two consecutive platelet transfusion episodes, has several explanations (Table 23-7). Nonimmune, patient-dependent factors are probably the most important, which means that poor platelet count recoveries can persist even when HLA alloimmunization is prevented with

**Table 23-7.** General Causes of Platelet Transfusion Refractoriness, Listed in Probable Descending Order of Frequency\*

- 
- Nonimmune mechanisms
  - Septicemia, fever, disseminated intravascular coagulation, amphotericin B therapy, hypersplenism, fixed platelet count requirements in severe thrombocytopenia
  - Platelet-nonspecific alloantibodies
    - HLA alloantibodies
    - ABO alloantibodies
  - Platelet-specific alloantibodies
  - Drug-dependent antibodies (eg, vancomycin)
  - Platelet-reactive antibodies
- 

\*Modified with permission from Warkentin & Smith.<sup>197</sup>

leukocyte-reduced blood components<sup>235</sup> and when HLA- or ABO-compatible platelets are given.

There is anecdotal evidence that platelet-specific alloantibodies sometimes cause refractoriness. However, prospective studies have shown that this is a relatively infrequent occurrence. For example, Novotny et al<sup>236</sup> found that even when HLA alloantibody formation was largely prevented with blood components filtered before storage, platelet-specific alloantibodies at most explained 4 of 79 (5%) of cases of refractoriness. There are occasions, however, on which the transfusion service needs to provide HLA- and platelet-specific antigen-compatible platelet products to manage certain of these patients.<sup>237</sup>

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## Summary

A variety of platelet-antibody and other assays have improved the ability of the clinician to make an accurate diagnosis of immune thrombocytopenia in many diverse clinical settings that can involve pathogenic autoantibodies, alloantibodies, and drug-dependent antibodies. The treatment decisions that arise depend upon several relevant factors, including the nature of the specific diagnosis, the expected prognosis, and the presence of clinically evident bleeding or thrombosis.

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# Bleeding from Acquired Coagulation Defects and Antithrombotic Therapy

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Acquired disorders of hemostasis may occur in association with specific clinical conditions or may arise spontaneously in otherwise healthy patients. Unlike inherited coagulation disorders, acquired coagulation disorders are often diagnosed in adult patients, although they may present at any age. Bleeding symptoms can vary from mild bruising and mucosal bleeding to prolonged postoperative bleeding and severe hemorrhage. If the patient does not have a history of significant hemostatic challenge such as surgery or trauma, it may be difficult to distinguish an acquired coagulation defect from an undiagnosed congenital disorder such as mild hemophilia or von Willebrand disease. Management of acute bleeding in patients with acquired coagulation disorders requires prompt recognition of the specific hemostatic defect to guide therapy. The initial laboratory evaluation should usually include the prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time, fibrinogen level, and platelet count. In most patients, the bleeding time test is not recommended, because it is not informative about the risk of bleeding or the proper treatment. Automated hemostatic assays, including use of the platelet function analyzer (PFA-100, Dade Behring, Deerfield, IL) and thromboelastography, are receiving increasing attention as potentially more informative alternatives to the bleeding time test.<sup>1,2</sup>

The acquired coagulation defects discussed in this chapter, and their characteristic effects on laboratory tests of hemostasis, are summarized in Table 24-1. Congenital disorders of coagulation are considered in Chapter 28, and hemostatic support in the perioperative setting and solid organ transplantation are reviewed in Chapters 38 and 40, respectively.

## Liver Disease

### Pathophysiology

Liver disease and liver transplantation are associated with multiple hemostatic defects, including quantitative and qualitative abnormalities of coagulation factors, thrombocytopenia, and platelet dysfunction. The liver produces most of the soluble components of the coagulation pathways and many of the regulatory proteins, including Factors II, V, VII, IX, X, XI, XII, and XIII, as well as prekallikrein, high-molecular-weight kininogen, plasminogen,  $\alpha_2$ -macroglobulin, antiplastins, antithrombin, thrombin activatable fibrinolysis inhibitor, tissue factor pathway inhibitor, ADAMTS13 metalloprotease, protein C, and protein S.<sup>3,4</sup> Factor VIII, the factor that is deficient in patients with classical hemophilia, is also produced by the liver. However, plasma levels of Factor VIII are determined by release of von Willebrand factor (vWF) from endothelium; therefore, levels may be elevated, rather than decreased, in patients with liver disease. The liver also plays a key role in clearance of activated coagulation factors and fibrinolytic fragments that interfere with hemostatic mechanisms. Significant liver dysfunction can, therefore, lead to bleeding from impaired biosynthesis of multiple hemostatic factors combined with failed clearance of inhibitory factors.

In both acute and chronic liver disease, the extent of hepatocellular damage generally correlates with the magnitude of the hemostatic defect, as assessed by laboratory tests and clinical bleeding. In acute hepatitis, plasma levels of the vitamin-K-dependent Factors II, VII, IX, and X are quantitatively decreased. Deficiency of Factor VII usually precedes that of other factors because of its short (6-hour) biological half-life. Levels of Factor IX tend to be less depressed than other vitamin-K-dependent factors. Levels of Factor V are variably decreased in acute hepatitis and are often decreased in chronic liver disease. Levels of fibrinogen are usually elevated in acute hepatitis as part of the acute phase response. In chronic hepatitis, levels of fibrinogen are typically

**Table 24-1.** Laboratory Tests of Hemostasis in Patients with Acquired Coagulation Defects

| Disorder or Therapy                            | PT  | aPTT | TT | Fibrinogen | Platelet Count |
|------------------------------------------------|-----|------|----|------------|----------------|
| Liver disease                                  | ↑   | (↑)  | ↑  | (↓)        | (↓)            |
| Vitamin K deficiency or warfarin therapy       | ↑   | ↑    | N  | N          | N              |
| Disseminated intravascular coagulation         | ↑   | ↑    | ↑  | ↓          | ↓              |
| Inhibitor to Factor VIII (acquired hemophilia) | N   | ↑    | N  | N          | N              |
| Acquired von Willebrand syndrome               | N   | (↑)  | N  | N          | N              |
| Inhibitor to Factor V                          | ↑   | ↑    | N  | N          | N              |
| Acquired Factor X deficiency                   | ↑   | ↑    | N  | N          | N              |
| Lupus anticoagulant                            | (↑) | (↑)  | N  | N          | N              |
| Heparin therapy                                | N   | ↑    | ↑  | N          | N              |
| Direct thrombin inhibitor therapy              | ↑   | ↑    | N  | N          | N              |
| Fibrinolytic therapy                           | ↑   | ↑    | ↑  | ↓          | N              |

PT = prothrombin time; aPTT = activated partial thromboplastin time; TT = thrombin time. Test results are indicated as normal (N), increased (↑), or decreased (↓). Parentheses indicate a mild or variable effect.

normal or mildly decreased, with levels less than 100 mg/dL indicative of end-stage liver disease and poor prognosis.

Biosynthesis of dysfunctional hemostatic proteins also contributes to the bleeding diathesis of liver disease. Impaired vitamin K utilization results in the production of dysfunctional, noncarboxylated vitamin-K-dependent factors. Production of abnormal fibrinogens (dysfibrinogenemia) and accumulation of fibrin degradation products (FDPs) can impair coagulation. Dysfibrinogenemia caused by abnormal posttranslational processing of fibrinogen is common in chronic active hepatitis and cirrhosis. The abnormal fibrinogens have been shown to have antithrombin activity and to impair fibrin polymerization, which may result in the formation of fibrin clots with impaired stability. Elevated FDP levels are caused by disseminated intravascular coagulation (DIC) and impaired hepatic clearance of FDPs.

Thrombocytopenia and platelet dysfunction are important components of the hemostatic deficit in liver disease. In chronic liver disease, portal hypertension with resultant splenomegaly and sequestration of platelets is the primary cause of thrombocytopenia. In acute viral hepatitis thrombocytopenia is usually modest, although platelet-dependent hemostatic mechanisms may be defective even when the platelet count is normal.

### Laboratory Features

The PT is the most sensitive laboratory screening test for coagulation defects in liver disease. The PT is particularly sensitive to decreased levels of Factor VII because of its short half-life. Factor VII is a reliable indicator of hepatic synthesis of vitamin-K-dependent hemostatic factors. The aPTT and thrombin time are also prolonged because of deficiencies of multiple coagulation factors and the inhibitory effects of dysfunctional fibrinogens and FDPs. When the thrombin time is prolonged, despite normal levels of fibrinogen and minor elevations of FDPs, dysfibrinogenemia should be suspected. In acute hepatitis, the fibrinogen level is usually normal, but may be

elevated when there is an acute phase response. A significant decline in plasma fibrinogen occurs only when acute liver disease is severe.

Prolonged clotting times usually correct to normal when the patient's plasma is mixed 1:1 with normal plasma. Levels of individual vitamin-K-dependent coagulation factors, as well as the non-vitamin-K-dependent Factor V, are often decreased in liver disease. In contrast, levels of Factor VIII are typically normal or elevated. It is usually not necessary to measure coagulation factors in patients with liver disease. However, measurement of Factors V, VII, and VIII can be useful in some patients to help distinguish between vitamin K deficiency (in which Factor VII is decreased and Factors V and VIII are normal), synthetic liver dysfunction (in which Factors V and VII are decreased and Factor VIII is normal or elevated), and consumption of coagulation factors (in which all three factors are generally decreased).

### Management of Bleeding

Bleeding associated with severe liver disease is a major clinical challenge requiring surgical, pharmacologic, and transfusion support. Because the hemostatic defect invariably includes deficiency of coagulation factors, often with coexisting thrombocytopenia, transfusion of plasma and platelets are the mainstay of therapy. Plasma or platelets may be transfused to treat active bleeding when the international normalized ratio (INR) is greater than 1.5 or the platelet count is below 50,000/μL, respectively. The utility of transfusion of plasma when the INR is more modestly elevated (ie, between 1.1 and 1.4) is uncertain.<sup>5</sup> The INR is a very poor predictor of bleeding in conjunction with surgical procedures, and the prophylactic transfusion of plasma to correct modest elevations of the INR before procedures has not been demonstrated to have a significant impact on clinical outcomes.<sup>5</sup> The prophylactic transfusion of plasma to correct modest elevations of the INR or in advance of minor procedures should be discouraged.



In bleeding patients, a typical initial dose of plasma is 15 mL/kg, with doses ranging up to 30 mL/kg in severe liver disease. The hemostatic benefit of plasma transfusion is often quite transient, necessitating frequent dosing, which can cause volume overload. The PT is used to monitor efficacy and to determine requirements for repeated administration. The PT may remain prolonged by up to 3 seconds despite hemostatically adequate levels of vitamin-K-dependent factors. It is often difficult to correct such mild elevations of the PT, and attempts to do so may consume large quantities of plasma without success. This difficulty may be related to the short half-life of Factor VII, the poor recovery of Factor IX from transfused plasma, and the loss of transfused clotting factors into third space fluid. Prolongation of the PT by more than 6 seconds often indicates that multiple factors are deficient.

Fibrinogen levels are seldom decreased enough from liver dysfunction to cause major bleeding. However, severe hypofibrinogenemia (fibrinogen level less than 100 mg/mL) in a patient with active bleeding should be treated with cryoprecipitate. Each unit of cryoprecipitate contains 150 mg to 250 mg of fibrinogen. A typical initial dose of 10 to 15 units should increase the plasma concentration of fibrinogen by 50 to 75 mg/dL. In Europe, fibrinogen concentrates fractionated from plasma and treated to inactivate or remove virus are available for this purpose.

An actively bleeding patient with a platelet count below 50,000/ $\mu$ L generally should be treated with transfusion of platelets. As with transfused plasma, multiple physiologic factors often diminish the recovery of transfused platelets. Although liver disease can cause qualitative platelet dysfunction, the therapeutic role of platelet transfusion in the absence of thrombocytopenia is unproven.<sup>3</sup>

Vitamin K should be administered to most patients with coagulopathy and liver disease, but its effectiveness is often poor; even if the PT is shortened, clinical bleeding may persist. Desmopressin acetate (DDAVP), which stimulates release of vWF (and possibly Factor VIII) from endothelial cells, is sometimes used. However, Factor VIII and vWF are often elevated in liver disease, so the value of DDAVP is uncertain and has not been rigorously tested.<sup>6</sup>

An evolving approach to the rapid treatment of the coagulopathy of liver disease is the use of recombinant activated Factor VII (rFVIIa).<sup>7</sup> Doses of rFVIIa ranging from 5 to 120  $\mu$ g/kg have been used before liver biopsy, with prompt normalization of the PT occurring in most patients. However, in this and several other settings of hemostatic challenge in liver disease, normalization of the PT associated with the use of rFVIIa has not been demonstrated to prevent bleeding or improve clinical outcomes. Thus, the utility of rFVIIa in treating the coagulopathy of liver disease remains unproven.

Prothrombin complex concentrates, which contain vitamin-K-dependent coagulation factors, are gaining renewed interest for treating the coagulopathy of liver disease.<sup>8</sup> Older prothrombin complex concentrates were occasionally associated with serious thromboembolic complications, but newer formulations appear to be safer and have been used successfully to rapidly reverse the coagulopathy of liver disease.

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## Vitamin K Deficiency

### Pathophysiology

Vitamin K comprises a group of fat-soluble vitamins that are available from many dietary sources and from gastrointestinal flora. Because there are two independent sources of vitamin K, deficiency solely from inadequate dietary absorption or inadequate production by gastrointestinal flora is rare. Deficiency usually arises from disruption of both sources.

Vitamin K is a coenzyme in the hepatocellular pathway that synthesizes coagulation Factors II, VII, IX, and X, as well as the regulatory factors protein C, protein S, and protein Z. Vitamin K is required for the  $\gamma$ -carboxylation of several amino terminal glutamyl residues during posttranslational processing. The carboxylated glutamyl residues provide negative charge densities that allow formation of ionic bridges between the factors and phospholipid surfaces and also facilitate protein-protein interactions between coagulation factors. The ability of the vitamin-K-dependent clotting factors to assemble on anionic phospholipid surfaces, such as platelet membranes, is essential for normal hemostasis. In conditions of vitamin K deficiency, plasma levels of vitamin-K-dependent coagulation factors are quantitatively decreased, and production of dysfunctional noncarboxylated factors further contributes to impaired hemostasis.

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## Hemorrhagic Disease of the Newborn

Hemorrhagic disease of the newborn is a postnatal bleeding disorder caused by inadequate production of vitamin-K-dependent coagulation factors. A transient physiologic decrease in coagulation factors normally occurs during the newborn period because of synthetic limitations of the immature liver. With maternal or newborn vitamin K deficiency, plasma concentrations of vitamin-K-dependent factors can decline to levels that are inadequate to maintain hemostasis. Bleeding in hemorrhagic disease of the newborn can be severe and can include melena, intracranial hemorrhage, bleeding from circumcision, generalized ecchymosis, and intramuscular hemorrhage. Factors that predispose to hemorrhagic disease of the newborn include prematurity, delayed bacterial colonization of the gut, liver disease, inadequate maternal or infant vitamin K intake, and prenatal exposure to warfarin or anticonvulsant drugs. Because breast milk is a poor source of vitamin K, postnatal deficiency of vitamin K tends to be more severe in breast-fed infants who do not receive vitamin K supplementation.

The PT is prolonged in hemorrhagic disease of the newborn, and levels of all of the vitamin-K-dependent factors are decreased. Levels of fibrinogen and Factors V and VIII are usually normal, as is the platelet count. Treatment relies on administration of vitamin K<sub>1</sub> (phytonadione). Intramuscular injection of 0.5 to 1 mg of vitamin K<sub>1</sub> usually produces normal neonatal levels of vitamin-K-dependent factors within 24 hours. In cases of

severe hemorrhage, transfusion of plasma should be given along with vitamin K<sub>1</sub>. Treatment with vitamin K<sub>1</sub> may be less effective in premature infants because of liver immaturity. Caution should be used with administration of vitamin K<sub>1</sub> in very large doses, which can produce hemolysis, hyperbilirubinemia, and kernicterus.

Prophylactic administration of vitamin K<sub>1</sub> to newborns (1 mg parenterally or 2 mg orally) diminishes the transient decrease in vitamin-K-dependent factors and prevents hemorrhagic disease of the newborn. This practice is mandated by law in many countries, which accounts for the rarity of the disorder in the developed world.

### Other Causes of Vitamin K Deficiency

Malabsorption syndromes, including celiac disease, sprue, inflammatory bowel disease, and parasitic infestations, can impair absorption of dietary vitamin K. Absorption of vitamin K also can be impaired in severe biliary stasis or after ingestion of bile acid sequestering resins. Ingestion of high-dose aspirin, high-dose vitamin E, warfarin, or other anticoagulants is a common cause of vitamin K deficiency, either from decreased absorption, or vitamin K antagonism. Antibiotic therapy contributes to vitamin K deficiency by inhibiting the synthetic capacity of vitamin-K-producing bacteria. Certain antibiotics, such as cephalosporins that contain an N-methyl thiotetrazol ring, interfere directly with vitamin K activity. The combination of inadequate dietary intake of vitamin K and use of broad spectrum antibiotics is an insidious cause of vitamin K deficiency in hospitalized patients.

### Treatment

A single oral dose of 5 to 10 mg of vitamin K<sub>1</sub> usually restores adequate levels of vitamin-K-dependent coagulation factors within 24 hours. Larger doses may be required in patients with severe deficiency, particularly those associated with warfarin or other anticoagulants. In cases of ingestion of warfarin-like rodent poisons, doses of vitamin K<sub>1</sub> up to 100 mg daily (orally or intravenously) may be required because of the potency and extremely long biological half-lives of these poisons. If emergent reversal of the bleeding diathesis is required, transfusion of plasma (15 mL/kg) should raise levels of vitamin-K-dependent factors to 30% to 50% of normal. Both rFVIIa and prothrombin complex concentrates have been used successfully to urgently reverse the effects of vitamin K antagonists in small numbers of patients.<sup>9-11</sup> General guidelines for the reversal of warfarin anticoagulation are summarized below in the section on Antithrombotic Therapy.

## Disseminated Intravascular Coagulation

### Pathophysiology

DIC is a syndrome of diverse etiology that is characterized by pathologic activation of procoagulant and fibrinolytic pathways. Systemic activation of these pathways creates two seemingly

paradoxical clinical problems: tissue injury caused by disseminated microvascular thrombosis, and hemorrhage caused by consumption of coagulation factors and accelerated fibrinolysis. Some patients, such as those with DIC caused by meningococcemia, experience severe thrombosis of the skin and other organs but have little clinical evidence of bleeding. Other patients with DIC present with bleeding from surgical sites, severe ecchymosis, and diffuse oozing from phlebotomy sites and mucosal surfaces. Still other patients have clinical manifestations of both thrombosis and hemorrhage.

DIC is usually a secondary phenomenon, and the clinical entities that underlie its development are impressively diverse. They include obstetric accidents, intravascular hemolysis, sepsis, viral illnesses, crush injuries, burns, head injuries, autoimmune and inflammatory disorders, malignancies, toxins, and medications. The most common causes of DIC in the developed world are obstetric accidents and infection. Worldwide, venomous snake bite is estimated to be among the most common causes of DIC.

DIC often occurs in patients who have features of the systemic inflammatory response syndrome (SIRS). In SIRS, pathologic processes evoke the production of inflammatory mediators that stimulate expression of tissue factor and release of plasminogen activators from endothelial cells.<sup>12</sup> Tissue factor initiates coagulation pathways, leading to the production of thrombin, which in turn stimulates platelet aggregation, activation of coagulation Factors V and VIII, and cleavage of fibrinogen to form fibrin matrices. Generalized production of thrombin can lead to diffuse microvascular deposition of fibrin and subsequent organ failure. Thrombin is normally regulated by the thrombomodulin/protein C anticoagulant system, but this regulatory system is impaired in DIC because of downregulation of thrombomodulin and consumption of protein C. Plasminogen activators activate plasmin, which proteolytically cleaves both fibrin clots (fibrinolysis) and soluble fibrinogen (fibrinogenolysis). Generation of plasmin in the systemic circulation causes proteolytic consumption of fibrinogen and accumulation of FDPs, both of which contribute to bleeding in DIC.

Platelet activation and aggregation lead to thrombocytopenia and bleeding in DIC. Consumption of platelets in DIC can be extremely rapid, overwhelming the capacity of the marrow to replenish the circulating pool of platelets. In addition, partial activation of platelets and accumulation of FDPs may produce a functional defect in the platelets that remain in the circulation. Severe deficiency of the vWF-cleaving metalloprotease ADAMTS13 has been reported in DIC and may contribute to platelet microvascular thrombosis.<sup>13</sup>

### Clinical Features

DIC is a spectrum of disorders with acute and chronic manifestations. In fulminant acute DIC, thrombosis and hemorrhage often produce multi-organ failure manifested by renal, pulmonary, hepatic, cutaneous, and central nervous system dysfunction. Metabolic instability, hypotension, fever, proteinuria,

and hypoxia are common. Hematologic signs include generalized ecchymosis, petechiae, or skin necrosis, and bleeding from mucosal surfaces, venipuncture sites, and surgical sites. In DIC associated with meningococcemia, the appearance of rapidly progressing retiform skin lesions is a poor prognostic sign. The skin lesions in these patients are caused by widespread thrombosis of dermal and subdermal vessels. The thrombotic diathesis of DIC also can include significant large vessel thrombosis.

Chronic DIC reflects low-grade systemic activation of hemostatic pathways and is associated with underlying vascular disease, autoimmune disorders, chronic inflammatory disorders, malignancies, and chronic liver disease. Because hemostatic compensatory mechanisms usually keep pace with consumption, bleeding may be less prominent than thrombotic complications, except with severe thrombocytopenia. Clinical manifestations are variable and may include deep venous thrombosis or migratory thrombophlebitis as well as epistaxis and ecchymosis.

### Laboratory Features

Many laboratory tests have been developed to aid in the diagnosis of DIC. These tests include assays for activated coagulation factors, specific activation fragments of coagulation factors, complexes of hemostatic factors with inhibitors, fibrin monomer, and various products of fibrin degradation. Although these assays offer the potential to refine the diagnostic criteria and pathophysiologic understanding of DIC syndromes, a more commonly available set of laboratory tests suffices in most clinical circumstances.

The initial laboratory evaluation should include the PT, aPTT, platelet count, fibrinogen concentration, and a test for FDPs or D-dimer. Collectively, these assays reflect the degree of depletion of hemostatic factors and the extent of fibrinolytic activity. The PT and aPTT assess the collective function of coagulation factors and provide an indication of available hemostatic resources. A low or falling fibrinogen level in combination with other coagulation abnormalities is strong evidence for DIC. FDPs and D-dimer are almost always elevated in DIC because of accelerated fibrinolysis. FDP levels reflect both fibrinolysis and fibrinogenolysis, while D-dimer detects only fibrinolysis and is a more specific indicator of fibrin formation. FDPs and D-dimer do not distinguish between the systemic fibrinolysis of DIC and the localized fibrinolysis that occurs following trauma or surgical procedures. Thus these tests must be interpreted together with fibrinogen levels and other hemostatic assays.

In chronic DIC, consumption of coagulation factors is partially compensated by their increased hepatic synthesis. Levels of fibrinogen and Factor VIII are often elevated as acute phase reactants. The PT and aPTT are normal or shortened. Increased FDP and D-dimer levels are of particular value in the diagnosis of chronic DIC.

### Treatment

Treatment of DIC requires control of the triggering pathologic process. Management of bleeding and thrombosis is an

important adjunctive measure, and can provide time for definitive therapies, such as antimicrobial therapy, surgery, or cancer treatment, to become effective. Prevention of ischemic organ injury is of paramount concern. Other supportive measures may include volume expansion and correction of hypotension to improve microcirculation and optimize tissue oxygenation.

The use of heparin in DIC highlights the dilemma of a disorder that simultaneously produces bleeding and thrombosis. While it may be counterintuitive to consider using an anticoagulant in the face of pathologic bleeding, the devastating consequences of systemic thrombosis make heparin a rational treatment for DIC. Moreover, by slowing consumption of coagulation factors, heparin may paradoxically improve the bleeding diathesis. Heparin inhibits thrombin activity and slows the consumptive process while reducing microvascular fibrin deposition. Heparin has been found to prevent complications of DIC in certain syndromes such as DIC associated with acute promyelocytic leukemia or solid tumors, but its value in treating DIC in other clinical settings is less certain. The initial dose of heparin in DIC is usually relatively low (for example, 8 U/kg/hour by continuous intravenous infusion), and titrated according to the hemostatic response. Declining levels of FDPs and D-dimer, rising levels of fibrinogen, and shortening of the aPTT demonstrate the efficacy of heparin in slowing consumption of coagulation factors and decreasing fibrin formation.

Despite theoretical concerns about “feeding the fire” of thrombosis, transfusion of platelets, fibrinogen, and other coagulation factors can be lifesaving in some patients with severe bleeding caused by DIC. In patients with active bleeding or impending biopsy and laboratory evidence of severe consumption of fibrinogen (fibrinogen level less than 100 mg/dL) or other coagulation factors (PT greater than  $1.5 \times$  control value), transfusion of cryoprecipitate (0.2 unit/kg) or plasma (15 mL/kg) is indicated. It is important to remember that many patients who have definitive laboratory evidence of DIC may not have clinically significant thrombosis or hemorrhage. Such patients typically do not require any specific therapy other than treatment of the underlying process that is triggering DIC.

When bleeding associated with DIC is refractory to heparin and transfusion therapy, antifibrinolytic agents such as  $\epsilon$ -aminocaproic acid are occasionally used. In addition to stabilizing clots, antifibrinolytic agents also reduce FDP production. However, inhibition of fibrinolysis has the potential to exacerbate fibrin deposition, resulting in severe thrombotic complications. Therefore, antifibrinolytic agents should be reserved for life-threatening hemorrhage, and should be considered for use only in conjunction with heparin in the setting of DIC.

Potential newer therapeutic agents for DIC include antithrombin and activated protein C, as well as small molecules that directly inhibit thrombin or Factor Xa.<sup>14</sup> Antithrombin is a serine protease inhibitor that inactivates thrombin, Factor Xa, and other coagulation factors. Heparin is a cofactor for antithrombin, and if antithrombin becomes depleted in DIC, the effectiveness of heparin anticoagulation may be limited. Antithrombin has been reported to be beneficial as an adjunctive to heparin therapy in

DIC when its levels are depleted to less than 70% of normal.<sup>14,15</sup> Protein C is an anticoagulant agent that inhibits thrombin production by inactivating Factors Va and VIIIa. Protein C and activated protein C have shown efficacy in clinical trials of DIC associated with bacterial sepsis.<sup>16,17</sup> These new agents and others in development offer the promise of improved treatment of DIC in the future.<sup>14</sup>

## Coagulation Factor Inhibitors

Normal blood plasma contains several proteins that inhibit activated coagulation factors. These natural coagulation factor inhibitors include antithrombin, heparin cofactor II,  $\alpha_1$ -protease inhibitor,  $\alpha_2$ -macroglobulin, C1 inhibitor, plasminogen activator inhibitors, and tissue factor pathway inhibitor. They function to limit the extent of hemostatic and fibrinolytic reactions and thereby localize thrombi to sites of vascular injury.

In contrast to natural coagulation factor inhibitors, pathologic inhibitors are usually immunoglobulins that bind directly to coagulation factors and either inhibit their activity or increase their clearance. Pathologic inhibitors, also known as circulating anticoagulants, may occur as an immunologic response to coagulation factor therapy in patients with hereditary hemophilia, or may arise spontaneously as autoantibodies in patients without a history of abnormal hemostasis.<sup>18</sup> Patients who spontaneously develop inhibitors of coagulation Factor VIII often present with a severe bleeding diathesis, and are said to have “acquired hemophilia.” Spontaneous inhibitors of other coagulation factors are encountered less frequently, but also can cause abnormal bleeding. When a coagulation factor inhibitor is suspected, it is essential to distinguish between an inhibitor of a specific coagulation factor, which often causes bleeding, and a nonspecific inhibitor such as a lupus anticoagulant, which may predispose to thrombosis rather than bleeding.

### Factor VIII Inhibitors (Acquired Hemophilia)

Acquired hemophilia is a rare disorder that is caused by the spontaneous development of an autoantibody inhibitor of coagulation Factor VIII. The annual incidence has been estimated to be about one case per million.<sup>18</sup> Autoantibodies to Factor VIII occur mainly in adults; they may arise in the postpartum period or in association with immunologic disorders such as systemic lupus erythematosus, rheumatoid arthritis, inflammatory bowel disease, or lymphoproliferative disorders. Approximately 50% of cases occur in elderly patients without any underlying medical condition.

The characteristic clinical presentation of acquired hemophilia is the appearance of pathologic hemorrhage in a patient with no history of abnormal bleeding. Patients may present with rapidly enlarging ecchymosis, soft tissue hematoma, gross hematuria, hemarthrosis, or gastrointestinal bleeding. Bleeding is sometimes severe and life threatening. With no known history of a bleeding disorder, the presence of an inhibitor is often not

recognized before surgical procedures, and some patients are diagnosed only after experiencing excessive postoperative bleeding.<sup>18</sup>

The presence of a circulating inhibitor to Factor VIII can be readily detected in the hemostasis laboratory. Typically, the aPTT is prolonged, and it does not correct to the normal reference range when repeated on a 1:1 mixture of the patient's plasma with normal plasma. Occasionally, the 1:1 mixture must be incubated for up to 2 hours to allow the inhibitor to completely inactivate Factor VIII before the aPTT is performed. The PT is usually normal. The Factor VIII activity level is low (often less than 10% of normal), but levels of other coagulation factors (such as Factors IX, XI, and XII) are normal. If multiple coagulation factors are affected, a nonspecific inhibitor (lupus anticoagulant) should be suspected. The diagnosis of a specific Factor VIII inhibitor is confirmed by a quantitative assay of the inhibitor level (often expressed in “Bethesda units” or BU). One Bethesda unit is defined as the amount of inhibitor that neutralizes half the Factor VIII activity in a 1:1 mixture with normal plasma.

The natural history of acquired hemophilia is variable. In some cases, such as those that present in the postpartum period, the inhibitor disappears spontaneously within weeks to months.<sup>19</sup> In other cases, the inhibitor persists for many years. In patients with active bleeding, the immediate goal of treatment is to control acute hemorrhage (Table 24-2). Invasive procedures, intramuscular injections, and the use of antiplatelet agents should be avoided if possible. If the bleeding is mucosal, an antifibrinolytic agent such as  $\epsilon$ -aminocaproic acid should be given.

If the inhibitor level is low (less than 10 BU), large doses of human Factor VIII (starting dose of 100 to 150 U/kg, followed by continuous infusion of 10 U/kg/hour) can overwhelm the inhibitor. Many clinicians prefer to use recombinant human Factor VIII, rather than plasma-derived Factor VIII concentrates, to minimize exposure to multiple blood donors.<sup>18</sup> Factor VIII levels should be measured frequently to assess the response to

**Table 24-2.** Management of Acute Bleeding Episodes in Patients with Acquired Factor VIII Inhibitors

#### Low inhibitor level (<10 BU)

- Desmopressin acetate (DDAVP)
- Human Factor VIII concentrate
- Recombinant human Factor VIII

#### High inhibitor level (>10 BU)

- Recombinant human Factor VIIa
- Porcine Factor VIII concentrate
- Prothrombin complex concentrate
- Activated prothrombin complex concentrate
- Plasmapheresis or immunoabsorption, followed by human Factor VIII concentrate or recombinant human Factor VIII

#### Adjunctive measures

- Immobilization
- Compression
- Avoid aspirin and other antiplatelet agents
- $\epsilon$ -aminocaproic acid (if bleeding is mucosal)

treatment; the usual goal of Factor VIII replacement in a patient with active bleeding is to maintain a Factor VIII activity level that is greater than 50% of normal.

Patients with very low inhibitor levels (less than 3 BU) may respond to DDAVP, which acts by stimulating release of endogenous vWF and Factor VIII from endothelial storage sites. DDAVP is administered either intravenously or nasally, and can be repeated every 24 hours for up to 3 days. Side effects of DDAVP include fluid retention and hyponatremia; therefore, this drug should not be used in elderly patients with a history of cardiovascular disease. Repeated use of DDAVP for more than three doses may result in loss of its hemostatic effectiveness because of depletion of vWF from storage sites.<sup>6</sup>

If the inhibitor level is high (greater than 10 BU), the likelihood of achieving a therapeutic response to human Factor VIII is low, and alternative treatments should be considered. Plasmapheresis or immunoabsorption columns have been used to acutely decrease the plasma level of a circulating inhibitor to allow successful treatment with human Factor VIII.<sup>20</sup> Some patients respond well to porcine Factor VIII concentrate (starting dose of 50 to 100 U/kg), because most acquired inhibitors have low cross-reactivity with porcine Factor VIII. However, continuous or repeated use of porcine Factor VIII often results in the development of inhibitors of porcine Factor VIII, which limits therapeutic options for management of future episodes of bleeding. It is recommended, therefore, that use of this product be restricted to life- and limb-threatening emergencies. Other therapeutic options for the management of acute bleeding in patients with acquired hemophilia include rFVIIa, prothrombin complex concentrates, and activated prothrombin complex concentrates (also called Factor VIII inhibitor bypassing concentrates). These products contain activated coagulation factors that appear to bypass Factor-VIII-dependent clotting reactions. Because the goal of therapy is to bypass (rather than replace) Factor VIII, it is not necessary to measure Factor VIII levels when using these products. It is important to distinguish between prothrombin complex concentrates, which contain Factor VIII bypassing activity, and highly purified plasma-derived Factor IX concentrates or recombinant Factor IX (eg, BeneFIX, Wyeth BioPharma, Madison, NJ), which cannot be used to bypass Factor VIII inhibitors.

The long-term management of patients with acquired Factor VIII inhibitors should be directed toward eradication of the inhibitor. Although some inhibitors disappear spontaneously, even after many years, a trial of immunosuppressive therapy should be considered in most patients. Some patients respond to corticosteroids alone.<sup>18</sup> Other immunosuppressive treatments include cyclophosphamide, azathioprine, cyclosporin A, intravenous immunoglobulin, and rituximab.<sup>19</sup>

### Lupus Anticoagulants

Lupus anticoagulants are autoantibodies that nonspecifically inhibit phospholipid-dependent coagulation reactions *in vitro*. Although first recognized in patients with systemic lupus

erythematosus, nonspecific inhibitors are actually encountered more frequently in patients without lupus. Lupus anticoagulants are induced by certain medications, including procainamide, hydralazine, quinidine, and chlorpromazine, or may occur in association with human immunodeficiency virus infection. Lupus anticoagulants also arise spontaneously in patients who are otherwise healthy.

Lupus anticoagulants represent a subset of a larger group of “antiphospholipid” autoantibodies that recognize phospholipid-protein complexes. Antiphospholipid antibodies are usually either IgG or IgM; other immunoglobulin classes are rare. Lupus anticoagulants often prolong the aPTT, but rarely prolong the PT. The aPTT usually does not correct completely to normal when the patient’s plasma is mixed 1:1 with normal plasma, but correction in mixing studies can be observed with lupus anticoagulants of low titer or low avidity. Some lupus anticoagulants do not prolong either the aPTT or PT. Several alternative clotting assays, including the dilute Russell viper venom time and kaolin clotting time, have been developed as high-sensitivity screening tests for lupus anticoagulants. Regardless of whether the aPTT or one of the alternative assays is used to detect the lupus anticoagulant, a confirmatory test should be performed to establish that the inhibitor is phospholipid dependent.<sup>21</sup>

Paradoxically, although lupus anticoagulants prolong clotting times *in vitro*, they are often associated clinically with thrombosis rather than with bleeding. Thrombotic events in patients with lupus anticoagulants can be either venous (such as deep venous thrombosis and pulmonary embolism) or arterial (such as myocardial infarction, stroke, and peripheral arterial occlusion). A syndrome of severe microvascular thrombosis resulting in acute multi-organ failure (the “catastrophic antiphospholipid antibody syndrome”) has been described in some patients.<sup>22</sup> In addition to thrombosis, patients with lupus anticoagulants may experience pregnancy loss, thrombocytopenia, neurologic symptoms, and livedo reticularis. The mechanisms by which lupus anticoagulants predispose to these clinical conditions are incompletely understood, but may involve autoantibody-mediated activation of procoagulant cell surface receptors<sup>23</sup> or disruption of the anticoagulant properties of annexin A5.<sup>24</sup>

Individual patients with antiphospholipid antibodies usually have positive laboratory test results for lupus anticoagulants, anticardiolipin antibodies, anti- $\beta_2$ -glycoprotein I antibodies, or a combination. However, none of the currently available laboratory tests for antiphospholipid antibodies predict with certainty which patients have an increased risk for clinical complications, and it is likely that only a subset of individuals with abnormal test results are actually predisposed to thrombosis. Patients with lupus anticoagulants, either with or without anticardiolipin antibodies, have a higher risk for thrombotic complications than those with isolated anticardiolipin antibodies.<sup>25</sup> Women with persistence of antiphospholipid antibodies also have an increased risk for miscarriage and other complications of pregnancy.

Identification of a lupus anticoagulant in a bleeding patient is important for several reasons. First, because most lupus

anticoagulants do not cause a bleeding diathesis (with the rare exception of those associated with severe thrombocytopenia or hypoprothrombinemia; see below), a search for another abnormality of hemostasis should be undertaken. Second, recognition that prolongation of the aPTT is caused by a lupus anticoagulant may prevent inappropriate transfusion of plasma and other blood components. Third, all patients with lupus anticoagulants should be considered to be at high risk for thrombosis, particularly in the settings of surgery, trauma, or pregnancy.

Patients with lupus anticoagulants may have coexistent autoimmune thrombocytopenia. The thrombocytopenia is often mild, but can be associated with abnormal bleeding if the platelet count decreases to less than 50,000/ $\mu$ L. Another circumstance in which lupus anticoagulants are directly associated with increased risk of bleeding, rather than thrombosis, is the hypoprothrombinemia-lupus anticoagulant syndrome. Patients with this syndrome appear to have an autoantibody inhibitor that causes accelerated clearance of prothrombin from plasma.<sup>26</sup> The PT is typically prolonged out of proportion to the aPTT. The diagnosis can be confirmed by performing a specific assay for prothrombin (Factor II) activity or antigen. A prothrombin level below 20% of normal can produce severe hemorrhage. Treatment of bleeding in such patients is challenging. Transfusion of plasma may be ineffective. Treatment options for acute bleeding include intravenous IgG and rFVIIa.<sup>27,28</sup> Immunosuppressive therapy with corticosteroids, danazol, or rituximab may be effective for long-term management.<sup>29,30</sup>

In patients with venous or arterial thrombosis, anticoagulant therapy with heparin followed by long-term anticoagulation with warfarin is usually indicated. The presence of a lupus anticoagulant may interfere with monitoring of heparin using the aPTT, and alternative monitoring methods such as Factor Xa inhibition assays may be needed to measure the heparin level. Alternatively, fixed-dose low-molecular-weight (LMW) heparin can often be used without laboratory monitoring. Lupus anticoagulants also can influence the INR in patients who are treated with warfarin. In such patients, it may be necessary to monitor warfarin therapy by measuring the level of a specific vitamin-K-dependent factor (such as Factor II or X).<sup>31</sup>

### Acquired von Willebrand Syndrome

Acquired deficiency of vWF arises spontaneously in previously healthy individuals or occur in association with neoplastic, rheumatologic, or hematologic disorders. Up to 50% of patients have monoclonal gammopathies or lymphoproliferative disorders.<sup>32</sup> The clinical presentation of acquired von Willebrand syndrome is similar to that of congenital von Willebrand disease, except that it occurs in individuals with no personal or family history of abnormal bleeding. Symptoms vary from mild cutaneous and mucosal bleeding (ecchymosis, epistaxis, gingival hemorrhage, or menorrhagia) to severe, life-threatening hemorrhage. The diagnosis should be suspected in patients who have a prolonged bleeding time or a prolonged platelet function analyzer (PFA-100) closure time. The aPTT is either prolonged or normal.

Levels of vWF activity (ristocetin cofactor activity), vWF antigen, and Factor VIII are often depressed. Most cases are caused by autoantibody inhibitors of vWF. The inhibitors cause rapid clearance of vWF from the circulation, and inhibitory activity often cannot be demonstrated in the patient's plasma by mixing studies. Variant forms of acquired von Willebrand syndrome occur in some patients with solid tumors, particularly Wilms' tumor. In these patients, the deficiency of vWF is not mediated by autoantibodies, but is caused instead by absorption of vWF to malignant cells or cell products.<sup>33</sup> Selective loss of high-molecular-weight multimers of vWF can be seen in patients with aortic valve stenosis.<sup>34</sup>

Management of bleeding in patients with vWF inhibitors can be difficult. As in patients with other bleeding disorders, invasive procedures, intramuscular injections, and the use of antiplatelet agents should be avoided if possible. In patients with an underlying hematoproliferative disorder, treatment with chemotherapeutic or cytoreductive agents may lead to resolution of the long-term bleeding diathesis.<sup>32</sup> In patients with acute bleeding, therapeutic options include DDAVP or infusion of Factor VIII concentrates that contain large quantities of vWF [eg, Humate P (ZLB Behring, King of Prussia, PA); Koate-DVI (Bayer Corporation, West Haven, CT); Alphanate (Grifols, Los Angeles, CA)]. Although these partially purified Factor VIII concentrates are derived from pooled plasma, they are subjected to virus attenuation procedures during processing, so the risk of viral transmission is considered to be low. Highly purified Factor VIII concentrates or recombinant human Factor VIII cannot be used, because these products generally contain little or no vWF. Purified vWF concentrates are available in Europe. Cryoprecipitate can be given as a source of vWF, but its use is discouraged because of a greater risk of exposure to blood-borne pathogens. Replacement therapy may be only transiently effective, because of the short half-life of vWF caused by excessive clearance from the circulation. Antifibrinolytic agents such as  $\epsilon$ -aminocaproic acid may be beneficial, particularly in patients with mucosal bleeding. Treatment with rFVIIa may be efficacious in some patients with vWF inhibitors.<sup>35</sup> Administration of high-dose intravenous IgG (2 g/kg over 2 to 5 days) may result in a rapid increase in vWF levels. Repeated administration of intravenous IgG every 3 weeks may be effective in controlling chronic bleeding.<sup>36</sup>

### Inhibitors of Factor V

Autoantibody inhibitors of Factor V can arise in patients following surgery, transfusions, or antibiotic therapy. The majority of cases have been reported in patients who were exposed to topical hemostatic agents, such as bovine thrombin or fibrin sealant, that contain trace amounts of bovine Factor V. The inhibitor presumably arises as an alloantibody to bovine Factor V that cross-reacts with human Factor V.<sup>37</sup> Inhibitors to Factor V may occur after a single exposure to fibrin sealant, but reexposure appears to increase the likelihood of inhibitor development.<sup>38</sup> Typically, both the PT and aPTT are prolonged, and do

not correct to normal when performed on a 1:1 mixture of the patient's plasma with normal plasma. The thrombin time also is prolonged because of the presence of a coexisting inhibitor to bovine thrombin. The diagnosis can be confirmed by performing a quantitative assay of the Factor V inhibitor level. The clinical presentation is quite variable, ranging from apparently normal hemostasis to severe hemorrhage, possibly because of differential effects of the inhibitor on plasma and platelet pools of Factor V. Treatment options for acute bleeding are limited; some patients respond to transfusion of platelets, immunoadsorption, or plasmapheresis.<sup>39</sup> In a majority of patients, the inhibitor disappears within weeks to months, and immunosuppressive therapy does not appear to influence the time course of the disease.

Acquired Factor X Deficiency

Acquired deficiency of Factor X can occur in patients with primary (AL) amyloidosis. The deficiency arises from accelerated clearance of Factor X from the circulation resulting from adsorption of Factor X to amyloid fibrils.<sup>40</sup> Both the PT and the aPTT are prolonged, but unlike the situation in patients with circulating inhibitors, the PT and aPTT usually correct to normal when performed on a 1:1 mixture of the patient's plasma with normal plasma. The hemostatic defect in these patients is multifactorial; in addition to Factor X deficiency, patients with amyloidosis may have excessive fibrinolysis and amyloid infiltration of blood vessels. Management of bleeding in such patients is problematic. Replacement therapy in the form of plasma or prothrombin complex concentrate is often ineffective, and antifibrinolytic agents such as ε-aminocaproic acid are of limited benefit.

Other Coagulation Factor Inhibitors

Autoantibody inhibitors of other coagulation factors are rarely encountered. As with inhibitors of Factor V, inhibitors of prothrombin prolong both the PT and the aPTT. Inhibitors of Factors IX or XI prolong only the aPTT. Specific factor and inhibitor assays can be performed to distinguish the type of inhibitor present. Depending on the inhibitor, treatment options for bleeding episodes include plasma, prothrombin complex concentrates, Factor IX concentrates, or rFVIIa. Adjunctive treatment with antifibrinolytic agents such as ε-aminocaproic acid also may be beneficial.

Inhibitors of Factor XIII do not affect either the PT or aPTT; specific assays of fibrin stabilization (such as the urea clot lysis assay) are necessary to identify these inhibitors.<sup>41</sup> In patients with acute bleeding, large doses of cryoprecipitate or Factor XIII concentrate can be given to try to overwhelm the inhibitor, but this approach may have limited effectiveness.

Acquired Platelet Function Disorders

Acquired disorders of platelet function occur much more frequently than congenital platelet abnormalities. Acquired platelet dysfunction is caused by drugs such as aspirin, medical

conditions such as chronic renal insufficiency, or procedures such as cardiopulmonary bypass. The risk of bleeding in patients with acquired platelet dysfunction is variable and unpredictable, and abnormal bleeding generally occurs only in the presence of additional hemostatic defects. Signs of platelet dysfunction manifest typically as mucocutaneous bleeding with excessive bruising, gingival bleeding, or epistaxis. Diagnostic tests of platelet function include the bleeding time and the platelet function analyzer (PFA-100). Although useful as screening tests, these tests lack diagnostic specificity, and they cannot be used to predict bleeding risk. The bleeding time may be prolonged because of abnormalities of cutaneous connective tissue, and both the bleeding time and the PFA-100 test results may be abnormal in the presence of anemia (hematocrit less than 30%) or thrombocytopenia (platelet count less than 100,000/ $\mu$ L). Many medications can alter platelet function and lead to a delayed PFA-100 closure time or prolonged bleeding time, so a thorough medication history must be obtained before these tests are performed. Formal platelet aggregation testing may be useful in the evaluation of selected patients.

Drug-Induced Platelet Dysfunction

A large number of drugs and medications have been reported to impair platelet function (Table 24-3). Among the most frequently encountered are aspirin, nonsteroidal anti-inflammatory drugs, and antiplatelet agents such as ticlopidine, clopidogrel, and abciximab. The antiplatelet effects of aspirin are irreversible, so recovery of normal hemostasis relies on the release of new platelets into the circulation rather than the disappearance of the drug from the plasma. The ability of platelets to aggregate is partially restored within 4 to 5 days after aspirin ingestion is stopped, but platelet dysfunction may persist for up to 7 days after aspirin is discontinued.<sup>42</sup> If possible, aspirin should be discontinued 7 to 10 days before a surgical or endoscopic procedure, unless it has been prescribed for the secondary prevention of stroke or myocardial infarction.<sup>43</sup> Most other nonsteroidal anti-inflammatory drugs inhibit platelet reactivity in a reversible manner, and platelet function usually returns to normal within 24 hours after the

Table 24-3. Some Drugs that Cause Impairment of Platelet Function

|                                      |                      |
|--------------------------------------|----------------------|
| Aspirin                              | Nitroglycerin        |
| Nonsteroidal anti-inflammatory drugs | Isosorbide dinitrate |
| Ticlopidine                          | Nitroprusside        |
| Clopidogrel                          | Nitroglycerin        |
| Abciximab                            | Isosorbide dinitrate |
| Eptifibatide                         | Nitroprusside        |
| Tirofiban                            | Nitroglycerin        |
| $\beta$ -Lactam antibiotics          | Isosorbide dinitrate |
| Prostacyclin                         | Nitroprusside        |
| Dipyridamole                         | Nitroglycerin        |
| Dextrans                             |                      |

drug is discontinued. Nonsteroidal anti-inflammatory drugs that selectively inhibit cyclooxygenase-2, such as celecoxib, appear to have very little effect on platelets and can be used to treat pain in patients with preexisting bleeding disorders such as hemophilia. The risk of clinical bleeding caused by aspirin or other nonsteroidal anti-inflammatory agents is generally low, and platelet transfusions should not be given prophylactically. However, ingestion of these drugs can increase the risk for serious bleeding in patients who also have additional hemorrhagic risk factors. If severe hemorrhage caused by defective platelet function is suspected, transfusion of platelets can rapidly restore normal hemostasis.

The thienopyridines, ticlopidine and clopidogrel, are platelet adenosine diphosphate (ADP)-receptor antagonists that inhibit platelet function by disrupting interactions with ADP and fibrinogen. Similar to aspirin, these drugs produce a persistent antiplatelet effect that lasts for up to a week after the drug is discontinued.<sup>42</sup> Therefore, these drugs usually should be discontinued 5 to 7 days before invasive procedures.

Abciximab is the prototype of a class of antiplatelet drugs that directly block the binding of fibrinogen to its platelet receptor, glycoprotein (GP) IIb/IIIa (integrin  $\alpha$ IIb $\beta$ 3). Other drugs in this class include eptifibatide and tirofiban. These agents are indicated for use in acute coronary syndromes and to prevent thrombosis of intravascular stents. They are often given in conjunction with other antithrombotic agents such as heparin.<sup>44</sup> Abciximab has an extended biological half-life because of its high affinity for GPIIb/IIIa; its antiplatelet effects can therefore persist for several days. In contrast, the antiplatelet effects of eptifibatide and tirofiban are transient, and usually resolve within a few hours of discontinuation in patients with normal renal function.<sup>45</sup> Management of bleeding in patients receiving GPIIb/IIIa antagonists may be complicated by the development of acute thrombocytopenia (platelet count less than 50,000/ $\mu$ L), which occurs in up to 5% of patients.<sup>46</sup> In cases of severe bleeding, platelet transfusions are an effective approach to control hemorrhage, either in the presence or absence of thrombocytopenia.

Many other drugs can impair platelet function (Table 24-3). The antiplatelet effects of  $\beta$ -lactam antibiotics are generally apparent only in patients receiving large parenteral doses of penicillins or cephalosporins. The frequency of clinically important bleeding in patients taking  $\beta$ -lactam antibiotics appears to be low, and is not predicted by the bleeding time or other laboratory tests of platelet function. Prostacyclin and dipyridamole inhibit platelet aggregation by elevating the intracellular concentration of cyclic adenosine monophosphate. Infusion of dextran inhibits platelet aggregation and enhances fibrinolysis through multiple mechanisms. Nitrovasodilators (nitroglycerin, isosorbide dinitrate, and nitroprusside) inhibit platelet function through nitric-oxide-dependent mechanisms. Both tricyclic antidepressants and selective serotonin reuptake inhibitors can alter platelet function, but the risk of serious clinical bleeding appears to be low.<sup>47</sup> Phenothiazines such as chlorpromazine, promethazine, or trifluoroperazine also have been reported to have mild antiplatelet effects. Consumption of ethanol can produce platelet

dysfunction as well as thrombocytopenia, and may contribute to clinical bleeding in patients with alcoholic liver disease.

## Uremia

Chronic renal insufficiency is associated with both hemorrhagic and thrombotic manifestations. Bleeding often manifests as ecchymoses, epistaxis, or gastrointestinal or genitourinary bleeding. The primary hemostatic abnormality in uremia is thought to be a defect in platelet function, but the pathophysiology of the platelet function defect remains poorly understood. Both dialyzable and nondialyzable substances contribute to the defect. As in many other acquired disorders of platelet function, the clinical importance of platelet dysfunction in uremia is uncertain. The bleeding time and PFA-100 closure time are prolonged, and platelet aggregation studies reveal defects in aggregation with ADP and epinephrine. In general, abnormalities of the bleeding time or platelet aggregation responses do not correlate with the severity of renal insufficiency or the degree of bleeding.

Management of bleeding in uremia is directed by the clinical circumstances rather than the results of laboratory tests of platelet function. Many patients with chronic renal failure do not have significant problems with bleeding despite a prolonged bleeding time and abnormal platelet aggregation responses. If bleeding is encountered in a patient with uremia, assessment of hemoglobin and hematocrit, platelet count, PT, and aPTT is necessary to evaluate for other potential causes of defective hemostasis. Coexisting anemia contributes to a bleeding propensity, and patients with bleeding may benefit from transfusion of red cells, or treatment with erythropoietin or darbopoietin, to maintain the hematocrit above 30%. Treatment with DDAVP or estrogens improves the bleeding time and prevents clinical bleeding in some patients with uremia.<sup>48</sup> Transfusion of cryoprecipitate may partially correct the hemorrhagic diathesis of uremia. Transfusion of platelets is usually not recommended in the absence of thrombocytopenia (platelet count less than 50,000/ $\mu$ L), because uremic plasma can induce dysfunction in transfused platelets. Patients with uremia may be unusually sensitive to anticoagulants or antiplatelet medications such as aspirin, which should be discontinued if possible. The benefit of intensive dialysis in correcting abnormal platelet function and diminishing bleeding is uncertain.<sup>48</sup>

## Cardiopulmonary Bypass

Abnormal platelet function, often in conjunction with thrombocytopenia, is a frequent cause of bleeding in patients undergoing cardiopulmonary bypass. The risk of perioperative bleeding varies depending on the type of surgical procedure, the age of the patient, preoperative renal function, and the duration of bypass. Prior exposure to antiplatelet agents and other antithrombotic medications increases the risk of bleeding.

The platelet defect caused by cardiopulmonary bypass is thought to result from activation of platelets within the extracorporeal circulation. Platelet activation is stimulated by multiple mediators, including thrombin generated from activation of the intrinsic and extrinsic coagulation pathways, mechanical stress,



complement activation, hypothermia, and exposure of platelets to the blood/air interface.<sup>49</sup> Partial activation and degranulation leads to desensitization and decreased adhesiveness of residual circulating platelets. The severity of platelet dysfunction correlates with the duration of bypass. Platelet function abnormalities usually resolve within 5 hours, but can persist for 24 hours or longer following complicated surgery.<sup>50</sup> Bleeding from platelet dysfunction is often exacerbated by thrombocytopenia caused by hemodilution and consumption of platelets. Consumption of coagulation factors, increased fibrinolytic activity, and inadequate neutralization of heparin with protamine sulfate also contribute to bleeding.

Management of bleeding associated with cardiopulmonary bypass is generally based on clinical considerations rather than laboratory testing. Bleeding times and PFA-100 closure times are frequently prolonged but are not predictive of perioperative blood loss.<sup>50</sup> Routine prophylactic administration of plasma or platelets is discouraged.<sup>51</sup> In the setting of excessive perioperative or early (within 24 hours) postoperative bleeding, however, platelet transfusions may be indicated even in the absence of severe thrombocytopenia.

Several drugs have been used to decrease the risk of perioperative bleeding during cardiopulmonary bypass. These include DDAVP, antifibrinolytic agents such as  $\epsilon$ -aminocaproic acid or tranexemic acid, and the protease inhibitor aprotinin. Some recent clinical trials have raised concern that the use of aprotinin might be associated with an increased risk of long-term morbidity and mortality.<sup>52</sup>

## Antithrombotic Therapy

Use of antithrombotic drugs has increased in recent years because of growing recognition of the efficacy of these medications for the prevention and treatment of venous thromboembolism, embolic stroke, and other thrombotic disorders. Although warfarin, heparin, and aspirin continue to be the most commonly prescribed antithrombotic medications, several new antithrombotic drugs are making their way into routine clinical use. These new drugs include LMW heparins, direct thrombin inhibitors, fibrinolytic agents, pentasaccharides, clopidogrel, and platelet GPIIb/IIIa antagonists.<sup>53</sup> The major complication of all these medications is bleeding. In this section, the management of bleeding associated with warfarin, heparin, LMW heparins, pentasaccharides, direct thrombin inhibitors, and fibrinolytic therapy is considered. Management of bleeding associated with antiplatelet medications is discussed above in the section on Drug-Induced Platelet Dysfunction.

### Warfarin

Warfarin and other vitamin K antagonists produce their antithrombotic effects by inhibiting the hepatic synthesis of vitamin-K-dependent coagulation factors. Vitamin K becomes oxidized to vitamin K epoxide during the  $\gamma$ -carboxylation of coagulation

Factors II, VII, IX, and X. Warfarin inhibits the cyclic regeneration of active vitamin K by blocking the action of vitamin K epoxide reductase and vitamin K reductase.<sup>54</sup> In the absence of adequate concentrations of active vitamin K, hepatic production of normally carboxylated factors is limited, and the liver instead produces hypocarboxylated factors with reduced coagulant activity.

Warfarin has a narrow therapeutic range with wide inter-individual dosing requirements. Its metabolism is influenced by diet, patient compliance, genetic factors, liver dysfunction, and interactions with other medications. Numerous drugs influence the pharmacokinetics of warfarin by altering its absorption or metabolic clearance, or by altering the production of vitamin K by intestinal flora. As a general rule, any change in medication regimen, including nonprescription medications and dietary supplements that contain vitamin K, should be presumed to have a potential effect on the anticoagulant response to warfarin. The risk of bleeding is highest during the initial phase of anticoagulation, within the first few weeks after initiation of therapy. Dosing algorithms that consider multiple parameters, such as gender, age, weight, height, race, and use of other medications, offer promise for decreasing the risk of early bleeding. Newer dosing algorithms incorporate pharmacogenomic information derived from genotyping two polymorphic genes (*CYP2C9* and *VKORC1*) that are involved in warfarin metabolism.<sup>55</sup> The clinical utility of these pharmacogenomic approaches is being tested in several clinical trials.<sup>56</sup>

The most common laboratory method for monitoring the anticoagulant effect of warfarin is the PT, often reported as the INR. Because the response of the PT to depletion of vitamin-K-dependent coagulation factors is highly variable when measured in different laboratories, the INR has been widely adopted as a method to standardize monitoring of oral anticoagulant therapy. The INR compares the ratio of the patient's PT to the mean PT of a group of normal individuals. The ratio is adjusted for the sensitivity of the PT reagent used in each laboratory. The target therapeutic INR for most indications is 2.0 to 3.0, although different therapeutic ranges are recommended for some indications.<sup>54</sup>

The risk of major bleeding in patients on chronic warfarin therapy has been estimated to be 1% to 5% per year.<sup>57</sup> Intensity of anticoagulation is probably the most important risk factor for hemorrhage, particularly when the INR is greater than 5.0. Clinical conditions that increase risk of hemorrhage include advanced age, hypertension, cerebrovascular disease, heart disease, renal insufficiency, a history of gastrointestinal bleeding, and concomitant use of antiplatelet agents. The risk of hemorrhagic complications resulting from warfarin therapy is decreased when patients are enrolled in coordinated programs for management of anticoagulation.<sup>58</sup>

Reversal of the anticoagulant effects of warfarin can be achieved through discontinuation of warfarin, administration of vitamin K<sub>1</sub>, transfusion of plasma or prothrombin complex concentrates, or treatment with rFVIIa. Management should be

guided by the degree of elevation of the INR and the presence or absence of clinical bleeding (Table 24-4). Minor or moderate elevation of the INR (above the therapeutic target but less than 9.0) in the absence of bleeding can often be managed safely by decreasing or omitting several doses of warfarin until the INR approaches the therapeutic range. Major elevation of the INR (greater than 9.0), even in the absence of clinically evident bleeding, should be treated by temporary discontinuation of warfarin and administration of vitamin K<sub>1</sub> (5 to 10 mg orally). Vitamin K<sub>1</sub> also can be administered intravenously, but intravenous administration is rarely associated with anaphylaxis. A recent meta-analysis found that oral and intravenous vitamin K<sub>1</sub> were equally effective in achieving a therapeutic INR at 24 hours in patients with excessive oral anticoagulation.<sup>59</sup> Subcutaneous administration of vitamin K<sub>1</sub> is associated with an unpredictable response and is therefore not recommended.<sup>59,60</sup> In patients with active major bleeding, administration of plasma (15 mL/kg) or prothrombin complex concentrates<sup>10</sup> may be lifesaving. Different preparations of prothrombin complex concentrates contain varying amounts of vitamin-K-dependent clotting factors and, therefore, differ in their clinical effectiveness for warfarin reversal.<sup>10</sup> Recombinant Factor VIIa is also used in such patients, especially

if there is life-threatening hemorrhage.<sup>9,54</sup> A dose of rFVIIa as low as 1.2 mg in an adult patient may be effective in normalizing the INR.<sup>9,61</sup> The clinical effectiveness of rFVIIa or prothrombin complex concentrates in the setting of warfarin-associated hemorrhage has not been subjected to randomized, controlled trials, however, and both preparations may be associated with an elevated risk of thromboembolic complications.<sup>62</sup>

Several approaches can be used to manage warfarin anticoagulation in patients who require elective surgery.<sup>54</sup> For many patients, a reasonable approach is to discontinue warfarin 4 to 5 days before surgery and begin heparin or a LMW heparin along with warfarin in the postoperative period. Heparin or LMW heparin can then be discontinued when the INR returns to the target therapeutic range. Patients who require major surgery urgently (within 24 hours) should receive plasma (15 mL/kg) and vitamin K<sub>1</sub> (2.5 to 5 mg orally or parenterally).

### Heparin

The antithrombotic effect of heparin is mediated by its ability to potentiate the inhibition of activated coagulation factors by antithrombin. In the presence of heparin, antithrombin irreversibly inactivates thrombin, Factor Xa, and to a lesser extent, other coagulation factors. Heparin is often administered intravenously, although it also may be given subcutaneously. When administered by intravenous infusion, the onset of its anticoagulant action is rapid.

Weight-based dosing of heparin appears to improve clinical outcomes in patients with venous thromboembolism.<sup>63</sup> A common dosing schedule utilizes an intravenous bolus dose of 80 U/kg followed by 18 U/kg/hour by continuous infusion. The dosage is then adjusted to maintain a therapeutic aPTT. The therapeutic range may vary between different laboratories that utilize different reagents for measuring the aPTT. In many laboratories, prolongation of the aPTT to a value that is 1.5- to 3.0-fold higher than the control value corresponds to a therapeutic heparin level. For certain indications, such as cardiopulmonary bypass surgery, higher doses of heparin are required and monitoring is performed using the activated clotting time rather than the aPTT.

The major side effect of heparin therapy is hemorrhage. In clinical studies of heparin administered for short-term treatment of venous thromboembolism, rates of major bleeding have ranged from 0% to 7%.<sup>57</sup> In some patients, bleeding is exacerbated by thrombocytopenia or platelet dysfunction. Because heparin has a short biological half-life of about 1 hour, the primary approach to management of bleeding is to discontinue the drug. In patients with therapeutic levels of heparin, recovery from its anticoagulant effects can be expected within 2 hours after discontinuation of heparin infusion. In cases of major hemorrhage, or when very large doses of heparin have been given (eg, for cardiopulmonary bypass or through a medication error), protamine sulfate should be administered.

Protamine sulfate is a strongly basic protein that neutralizes the anticoagulant effects of heparin within minutes. It is given by slow intravenous injection. The recommended dose of protamine

**Table 24-4.** Guidelines for Reversal of Warfarin Anticoagulation

| INR     | Bleeding | Recommendation                                                                                                                                                                                |
|---------|----------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <5.0    | None     | Decrease or omit warfarin dose                                                                                                                                                                |
|         | Minor    | Discontinue warfarin<br>Consider vitamin K <sub>1</sub> (2.5 mg orally)                                                                                                                       |
|         | Major    | Discontinue warfarin<br>Vitamin K <sub>1</sub> (10 mg by slow intravenous infusion*)<br>Fresh frozen plasma (15 mL/kg) or prothrombin complex concentrate<br>Consider recombinant Factor VIIa |
| 5.0-9.0 | None     | Omit one or two doses of warfarin; resume at lower dose                                                                                                                                       |
|         | Minor    | Discontinue warfarin<br>Vitamin K <sub>1</sub> (2.5 to 5.0 mg orally)                                                                                                                         |
|         | Major    | Discontinue warfarin<br>Vitamin K <sub>1</sub> (10 mg by slow intravenous infusion*)<br>Fresh frozen plasma (15 mL/kg) or prothrombin complex concentrate<br>Consider recombinant Factor VIIa |
| >9.0    | None     | Hold warfarin<br>Vitamin K <sub>1</sub> (5 to 10 mg orally)                                                                                                                                   |
|         | Minor    | Discontinue warfarin<br>Vitamin K <sub>1</sub> (5 to 10 mg orally)<br>Consider fresh frozen plasma (15 mL/kg)                                                                                 |
|         | Major    | Discontinue warfarin<br>Vitamin K <sub>1</sub> (10 mg by slow intravenous infusion*)<br>Fresh frozen plasma (15 mL/kg) or prothrombin complex concentrate<br>Consider recombinant Factor VIIa |

\*Intravenous administration of vitamin K<sub>1</sub> may produce (but rarely) an anaphylactic reaction.

INR = International Normalized Ratio

sulfate is 1.0 mg for every 100 units of heparin remaining in the patient, which can be calculated based on the 60-minute half-life of heparin. For example, a patient receiving 1000 units of heparin per hour by continuous intravenous infusion should be given enough protamine to neutralize all of the heparin administered within the last hour (1000 units), plus half of the heparin administered in the preceding hour (500 units), plus a quarter of the heparin administered in the hour prior to that (250 units). Therefore, the total dose of protamine sulfate would be 17.5 mg. Protamine sulfate itself has a weak anticoagulant effect, and over-dosage may exacerbate bleeding.

### Low-Molecular-Weight Heparins

LMW heparins are derived from heparin by chemical or enzymatic fragmentation.<sup>63</sup> Several LMW heparins, including dalteparin, enoxaparin, and tinzaparin, are available for prevention or treatment of venous thromboembolism, unstable angina, and other indications. Danaparoid sodium is not derived from heparin, but it is a mixture of LMW heparin-like glycosaminoglycans. Although danaparoid sodium is considered to be a “heparinoid,” its effects are very similar to those of the LMW heparins.

Compared with heparin, LMW heparins have less inhibitory activity against thrombin and greater relative inhibitory activity against Factor Xa. The LMW heparins produce a more predictable anticoagulant response than heparin, reflecting their better bioavailability, longer half-life, and dose-independent clearance. These properties allow LMW heparins to be given subcutaneously once or twice daily, usually without laboratory monitoring. Because LMW heparins are cleared through a renal route, dosages need to be adjusted in patients with renal insufficiency.

A large number of randomized clinical trials have demonstrated that LMW heparins are at least as safe and effective as heparin for most indications.<sup>63</sup> The incidence of heparin-induced thrombocytopenia is lower with LMW heparins than with heparin (see Chapter 23).

Despite the apparently favorable safety profile of LMW heparins, bleeding remains a major side effect. In clinical trials evaluating LMW heparins for treatment of venous thromboembolism, the rates of major bleeding have ranged from 0 to 3%.<sup>57</sup> Epidural bleeding and spinal hematoma have been reported in patients receiving LMW heparins concurrently with spinal or epidural anesthesia.<sup>64</sup> Management of bleeding is complicated by the long half-life of these medications. Unlike heparin, the anticoagulant effects of LMW heparins cannot be reversed completely by administration of protamine sulfate. Protamine sulfate can be administered in an attempt to control active bleeding in patients who have received LMW heparin within 8 hours, but large doses (20 to 50 mg) may be required.<sup>63</sup>

### Pentasaccharides

The pentasaccharides, fondaparinux and idraparinux, are synthetic analogs of the antithrombin-binding region of heparin.<sup>65</sup> These drugs selectively inhibit Factor Xa without having any appreciable

inhibitory effect on thrombin. Fondaparinux has a half-life of 18 to 22 hours and is usually administered subcutaneously once daily. It is used for the prevention and treatment of venous thromboembolism and in acute coronary syndromes. Idraparinux is an investigational pentasaccharide that has a substantially longer half-life of 130 hours and can be administered in a fixed dose once weekly.<sup>65</sup> The pentasaccharides have no specific antidotes, and their anticoagulant effects are not reversed by administration of plasma, prothrombin complex concentrates, or protamine sulfate.

### Direct Thrombin Inhibitors

The direct thrombin inhibitors include desirudin, lepirudin, bivalirudin, and argatroban. Because the direct thrombin inhibitors are structurally unrelated to heparin, one major indication for their use is in the treatment of heparin-induced thrombocytopenia. They also are being evaluated for use in prophylaxis of deep venous thrombosis, cardiopulmonary bypass surgery, and acute coronary syndromes.<sup>65</sup>

Most of the direct thrombin inhibitors are administered parenterally, and are monitored using the aPTT. They have short half-lives (less than 2 hours) when given by intravenous infusion. Because lepirudin, desirudin, and bivalirudin are cleared renally, their half-lives may be prolonged dramatically in renal failure. Clearance of argatroban is not influenced by renal impairment, but may be decreased in the presence of hepatic dysfunction. The risk of bleeding in patients receiving direct thrombin inhibitors appears to be dose-dependent.<sup>66</sup> Management of bleeding relies on prompt discontinuation of the drug.

### Fibrinolytic Agents

Pharmacologic lysis of fibrin thrombi is a commonly used strategy for treatment of acute myocardial infarction and stroke, and also is used in selected cases of peripheral arterial occlusion, and venous thromboembolism. Most fibrinolytic agents in clinical use are plasminogen activators, which include streptokinase, urokinase, and recombinant forms of tissue plasminogen activator such as alteplase and reteplase. Plasminogen activators produce thrombolysis by converting plasminogen to plasmin, which then degrades fibrin into soluble FDPs. Fibrinolytic agents can be administered systemically by intravenous infusion, or delivered in proximity to sites of thrombi via catheter-directed approaches. Contraindications to the use of fibrinolytic therapy include recent hemorrhagic stroke or major surgery, prolonged cardiopulmonary resuscitation, uncontrolled hypertension, and active gastrointestinal bleeding.<sup>67</sup>

In addition to producing therapeutic lysis of pathologic thrombi, fibrinolytic agents generate plasmin in the systemic circulation, resulting in bleeding from lysis of hemostatic plugs at surgical sites and other locations.<sup>67</sup> Bleeding, therefore, is a major complication of fibrinolytic therapy. Because circulating plasmin degrades fibrinogen, hypofibrinogenemia can contribute to bleeding. The extent of systemic fibrinogenolysis varies with different fibrinolytic agents and different routes of administration.<sup>68</sup> Laboratory abnormalities associated with the use of

fibrinolytic agents include decreased fibrinogen, decreased plasminogen, prolonged thrombin time, and decreased euglobulin clot lysis time. These laboratory parameters are poorly predictive of bleeding risk, however.<sup>67</sup>

The risk of major hemorrhage associated with fibrinolytic therapy is influenced by the age of the patient and concomitant use of additional antithrombotic agents such as heparin, direct thrombin inhibitors, or antiplatelet agents. In the absence of invasive procedures or simultaneous heparin therapy, treatment of acute myocardial infarction with fibrinolytic agents generally is associated with a low incidence of major bleeding (less than 5%). Intracranial hemorrhage is a rare, but potentially devastating, complication of fibrinolytic therapy. In large clinical trials of streptokinase, or alteplase for treatment of acute coronary ischemia, the incidence of intracranial hemorrhage has ranged from 0.2% to 0.8%.<sup>67</sup>

Bleeding that occurs in association with fibrinolytic therapy can often be managed by discontinuing infusion of the plasminogen activator and replacing fibrinogen by transfusion of cryoprecipitate (0.2 unit/kg) or (in Europe) by infusion of fibrinogen concentrates. The goal of transfusion therapy with cryoprecipitate is to maintain a plasma fibrinogen level of greater than 100 mg/dL. Adjunctive treatment with antifibrinolytic agents such as  $\epsilon$ -aminocaproic acid or aprotinin may be beneficial when rapid reversal of bleeding is desired.

## Disclaimer

The authors have disclosed no conflicts of interest.

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## 25

## Fetal and Neonatal Hematopoiesis

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The purpose of hematopoiesis in the fetus differs from that in the adult. The primary function of hematopoiesis in adults is to produce sufficient hemic cells to balance hemic cellular losses. In contrast, in the fetus, constant growth and dramatic physiologic changes necessitate a system with other functions. For example, the remarkable rate of somatic growth in the fetus and neonate and the resultant need to constantly increase blood volume necessitate an extraordinary hematopoietic effort, assessed as daily cell production per kilogram of body weight. In addition, the relatively low oxygen tensions but high metabolic rates of fetal tissues require a system of oxygen delivery fundamentally different from that in adults. Moreover, the sterile intraamniotic environment and consequently the low demand for the antimicrobial actions of neutrophils change markedly at birth. The extrauterine environment demands a constant and lifelong need for the antimicrobial actions of neutrophils. These issues are summarized in Table 25-1.

The hematopoietic system of a fetus and neonate must be sufficiently plastic to accommodate many marked changes and dichotomies. It has been speculated that improved familiarity with developmental hematopoietic regulation improves interpretation of postnatal hematologic data and enhances appreciation of the granulocytopenic, erythropoietic, and thrombopoietic capacities and limitations of prematurely delivered neonates. Cytopenia certainly is common in neonatal intensive care units (NICUs). Neutropenia occurs in 5% to 8% of patients in NICUs,<sup>1</sup> thrombocytopenia in 25% to 30%,<sup>2</sup> and anemia in perhaps as many as 50%<sup>3</sup> at some time before discharge. The prevalence of cytopenia among patients in NICUs is related to gestational age. The prevalence is higher among those delivered at the earliest gestational ages. Transfusion is the only means of managing severe cytopenia among neonates, but alternatives to

**Table 25-1.** Inherent Differences in Hematopoietic Systems of the Fetus, Neonate, and Adult

**Fetus**

- Because the fetus exists in a sterile environment, it does not generally need an antibacterial defense system until near term.
- In preparation for extrauterine life, the fetus must produce a neutrophil reserve.
- Fetal hematocrit and blood platelet concentration increase only slightly from 20 weeks to term, whereas blood volume increases approximately 10-fold. Erythrocyte and platelet production must be extremely rapid during this period to keep pace with the rapid expansion of blood volume.

**Neonate**

- At birth, the fetus moves from a sterile into a nonsterile environment and must have a neutrophil reserve already developed to survive in this environment.
- At birth, oxygen delivery to tissues markedly increases, as PaO<sub>2</sub> increases from 27 to >90 mm Hg. This effectively shuts off erythropoietin production. Consequently, erythropoiesis temporarily ceases, resulting in the physiologic anemia of infancy.
- Rapid growth and blood volume expansion continue. However, platelet concentration does not change, and platelet production is very rapid during this period.

**Adult**

- The rapid somatic growth and blood volume expansion of infancy and childhood cease. The previous need to accelerate hematopoiesis to keep pace with somatic growth ends.
- The neutrophil system must continue to be responsive to rapid increases in demand for cells.
- Blood platelet concentration and hematocrit remain relatively constant throughout healthy life.

repeated transfusions should emerge as more is learned about developmental hematopoiesis.

Developmental hematopoiesis can be viewed as occurring in three anatomic stages—mesoblastic, hepatic, and myeloid. Mesoblastic hematopoiesis occurs in extraembryonic structures, principally the yolk sac, and begins between the 16th and 19th days of gestation. By about 6 weeks of gestation, the extraembryonic sites of hematopoiesis begin to ablate and hepatic hematopoiesis is initiated. By the 10th to 12th week,

mesoblastic hematopoiesis ceases, and a small amount of hematopoiesis is evident in the marrow. In humans, it seems that the clavicle is the first bone to develop a marrow cavity.<sup>4</sup> The first cells present in the developing marrow space have macrophage surface markers and phenotypes. These are followed by myeloperoxidase-positive cells with the characteristics of neutrophils. However, the liver remains the predominant hematopoietic organ until the last trimester of pregnancy.

The anatomic site of hematopoiesis does not simply transfer from yolk sac to liver to marrow.<sup>5</sup> Rather, each organ subsequently houses distinct hematopoietic populations. For example, at 18 to 20 weeks of gestation, more than 85% of the cells in the fetal liver are erythroid, and few neutrophils are present. At the same time, fewer than 40% of the cells within the marrow are erythroid, and most are neutrophils. The mechanisms responsible for the changing anatomic sites of hematopoiesis and for the differences in hemic cells produced in the mesoblastic, hepatic, and myeloid sites have not been determined. Regardless of gestational age or anatomic location, production of all hematopoietic tissues begins with pluripotent cells capable of both self-renewal and clonal maturation into all blood cell lineages. Progenitor cells differentiate under the influence of hematopoietic growth factors, which include those listed in Table 25-2.

Granulocytopoiesis

Sites of Neutrophil Production in the Fetus

A traditional view is that neutrophil production, similar to hematopoiesis in general, begins in the yolk sac and moves to the liver and spleen and finally to the marrow. However, recent results of studies of human fetuses do not support this concept. Specifically, the human yolk sac contains no neutrophils—its hematopoietic activity is limited to erythropoiesis and production of a small number of macrophages.<sup>5</sup> Similarly, the human fetal liver produces few, if any, neutrophils. The few neutrophils found in photomicrographic sections of human fetal liver are not arranged in clusters of hematopoietic nests but are widely separated and found surrounding the blood vessels, as if they were carried in by the circulation, not produced in the organ. Moreover, the spleen is not a granulocytopoietic organ in human fetuses, as it is in rodents.<sup>6</sup> No granulocytopoietic nests are found in human fetal spleen, and the neutrophils within it are mostly mature and evenly dispersed. This finding suggests they were carried there in the blood, not produced locally.

Where, then, do neutrophils originate in the human fetus? The first neutrophils are present approximately 5 weeks after

Table 25-2. Hematopoietic Growth Factors

| Growth Factors             | Molecular Mass (kD) | Chromosomal Location | Principal Target Cell                 |
|----------------------------|---------------------|----------------------|---------------------------------------|
| Erythropoietin             | 30.4                | 7q11-22              | CFU-E, fetal BFU-E                    |
| Colony-stimulating factors |                     |                      |                                       |
| G-CSF                      | 18.8                | 17q11.2-21           | CFU-G                                 |
| GM-CSF                     | 14.4                | 5q23-31              | All CFC                               |
| M-CSF                      | 26 (dimer)          | 1p13-21              | CFU-M                                 |
| SCF                        | 15–20 (dimer)       | 12q2-24              | Primitive CFC                         |
| Interleukins               |                     |                      |                                       |
| IL-1 $\alpha$              | 17                  | 2q13                 | Primitive CFC, hepatocyte, macrophage |
| IL-1 $\beta$               | 17                  | 2q13                 | Primitive CFC, hepatocyte, macrophage |
| IL-2                       | 15-20               | 4q26-27              | T cell                                |
| IL-3                       | 14-15               | 5q23-31              | All CFCs                              |
| IL-4                       | 16-20               | 5q23-31              | T cell, B cell                        |
| IL-5                       | 13.2 (dimer)        | 5q23-31              | CFU-EOS                               |
| IL-6                       | 20.8                | 7p21-24              | Primitive CFC                         |
| IL-7                       | 25                  | 8q12-13              | B cell                                |
| IL-8                       | 8–10                | 4                    | Neutrophil, endothelial cell          |
| IL-9                       | 16                  | 5q31-32              | BFU-E, primitive CFC                  |
| IL-10                      | 23                  | 1                    | Macrophage, lymphocyte                |
| IL-11                      | 22                  | 19q13                | Primitive CFC, BFU-MK, CFU-MK         |
| IL-12                      | 70-75               |                      | T cell, NK cell, macrophage           |
| Thrombopoietin             | 35                  | 3q26-28              | BFU-MK, CFU-MK                        |

BFU-E = primitive erythroid progenitor; G-CSF = granulocyte colony-stimulating factor; CFU-G = colony-forming unit–granulocyte; GM-CSF = granulocyte-macrophage colony-stimulating factor; CFC = colony-forming cells; M-CSF = macrophage colony-stimulating factor; CFU-M = colony-forming unit–macrophage; SCF = stem cell factor; IL = interleukin; CFU-E = colony-forming unit–erythroid; CFU-EOS = colony-forming unit–eosinophil; BFU-MK = primitive megakaryocyte progenitor; CFU-MK = mature megakaryocyte progenitor; NK = natural killer.

conception and are clustered in the periaortic tissue. These first fetal neutrophils contain myeloperoxidase, and they mature into cells with segmented and band nuclei. But beyond these features, any differences or similarities between them and the neutrophils of adults have not been reported.<sup>7</sup> The function of these cells in the fetus and the explanation for this location of origin are unclear. The fetal marrow space begins to develop approximately 8 weeks after conception (Figs 25-1 and 25-2). The space is lined by osteoclast-like cells with cell-surface characteristics of macrophages. These cells appear to core out the space from the primitive cartilage. Eight to 10 weeks after conception, the marrow space progressively enlarges, but no neutrophils are present within the space until 10.5 to 11 weeks<sup>4</sup> (Fig 25-3). The first neutrophils in the marrow do not have segmented or band nuclei, but they contain myeloperoxidase and express the cell surface characteristics of myeloblasts and promyelocytes. From 14 weeks to term, the most common cell type in the fetal marrow space is the neutrophil, although the marrow space is not nearly so densely packed with cells as it becomes in older children and adults.<sup>4,7</sup>

Macrophages are crucial to fetal morphogenesis, because they aid in shaping of organs and scavenging debris and apoptotic cells. Although neutrophils and macrophages have a



**Figure 25-1.** Clavicle approximately 7 weeks after conception, before any marrow space is present. In the human fetus, the first bone to contain a developing marrow space is the clavicle, followed closely by the other long bones. (Hematoxylin and eosin stain; original magnification,  $\times 100$ ).

common progenitor cell, the discordant temporal appearance of neutrophils and macrophages in the fetus and the divergence of their anatomic locations are striking.<sup>8</sup> Recent observations have cast doubt on long-held theories about the origins of macrophages in the human fetus. For example, it was believed that macrophages form from precursor cells in the marrow and mature through a progression of cell types from monoblasts to promonocytes to monocytes, which then migrate to various tissues and differentiate into macrophages. Observations of human fetuses do not support this origin of macrophages. First, macrophages appear in the yolk sac, liver, lung, and brain long before the marrow cavity has been formed. Second, promonocytes and monocytes are absent in the yolk sac and liver, but macrophages are present there nevertheless. Thus, the macrophages in the yolk sac may develop directly from stem cells without passing through a monocyte stage. It is not clear whether these primitive macrophages migrate from the yolk sac to populate the lungs, liver, brain, and other organs.<sup>9</sup>

### Regulation of Neutrophil Production

The mechanisms that regulate neutrophil production during human fetal development are not clear. Granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF) are present in developing fetal bone as early as 6 weeks after conception and in the fetal liver as early as 8 weeks.<sup>10</sup> Granulocyte-macrophage colony-stimulating factor (GM-CSF) is widely distributed in fetal tissues, including pulmonary epithelium, and is involved in pulmonary homeostasis. Stem cell factor (SCF) messenger RNA (mRNA) is present in the yolk sac, liver, and marrow at the earliest stages of their respective development.<sup>5</sup> No changes in mRNA concentrations of these factors or of their specific receptors or in the concentrations of proteins, judged by immunohistochemical staining, appear to constitute the signal for production of neutrophils. The precise signals that initiate the production of macrophages and neutrophils in the embryo and fetus are not known. It is curious that the actions of G-CSF, M-CSF, GM-CSF, and SCF are not limited to hematopoiesis in the fetus and neonate. Receptors for all of these factors are located in distinct areas of the fetal central nervous system and gastrointestinal tract, where their patterns of expression change with development. Important undefined developmental roles clearly exist for these factors that are beyond those known for hematopoiesis. Although not fully characterized, these roles appear to be predominantly antiapoptotic, perhaps providing some degree of protection during adverse conditions such as hypoxemia or acidosis.

Although neutrophil production in the marrow space clearly is present by 14 weeks of gestation, the blood of the fetus, even through 20 weeks, contains few neutrophils. Forestier et al.<sup>11</sup> reported that fetuses at 20 weeks of gestation had a mean absolute blood neutrophil concentration of only  $190/\mu\text{L}$ , a range of 0 to  $490/\mu\text{L}$ , and a mode concentration of zero.<sup>11</sup> Despite the near absence of circulating neutrophils in the first trimester, and the relative scarcity (by adult standards) of neutrophils in the second



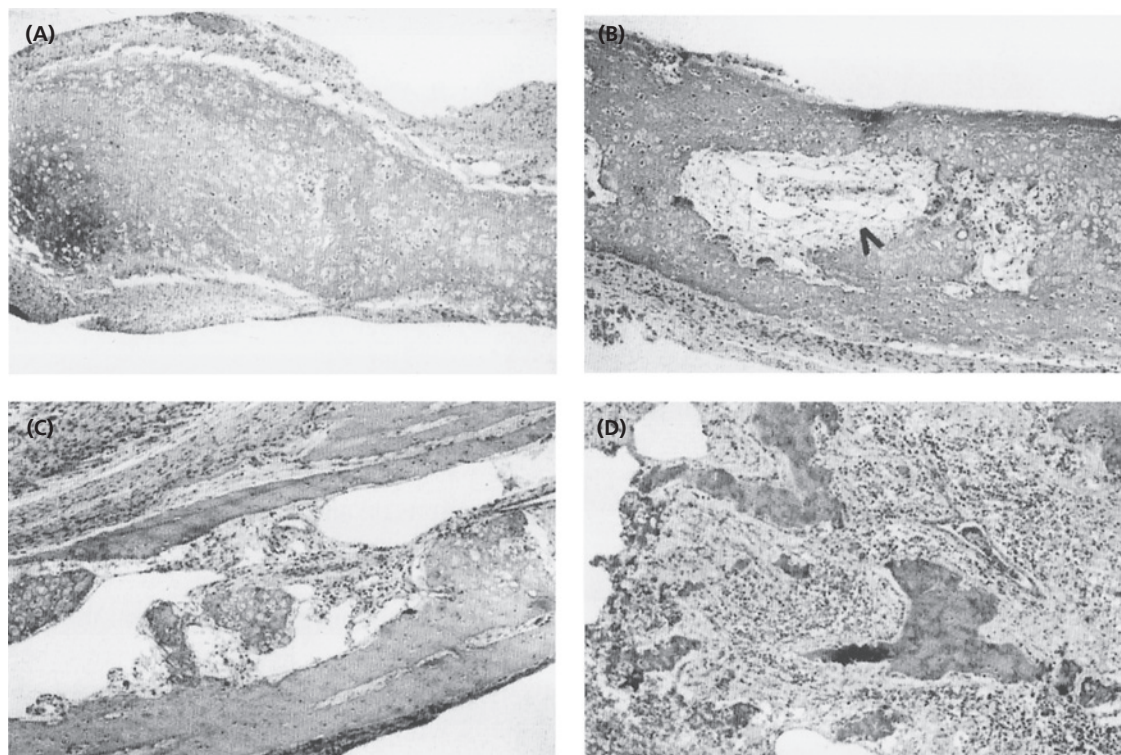


**Figure 25-2.** Photomicrographs of clavicles 6 to 14 weeks after conception (Hematoxylin and eosin stain; original magnification,  $\times 100$ ). (A) Six weeks after conception. Clavicles at this stage consist of primitive cartilage and contain no marrow cavity and no myeloperoxidase positive cells. (B) Nine weeks after conception. Clavicles at this stage have the beginning of a marrow cavity (*arrowhead*) but

no myeloperoxidase positive cells. (C) Eleven weeks after conception. Clavicles at this stage have an elongated marrow cavity and small hematopoietic islands of myeloperoxidase positive cell in the marrow space. (D) Fourteen weeks after conception. Spiculation has begun, and the volume of hematopoietic marrow has begun to increase. (Modified with permission from Slayton et al.<sup>4</sup>)

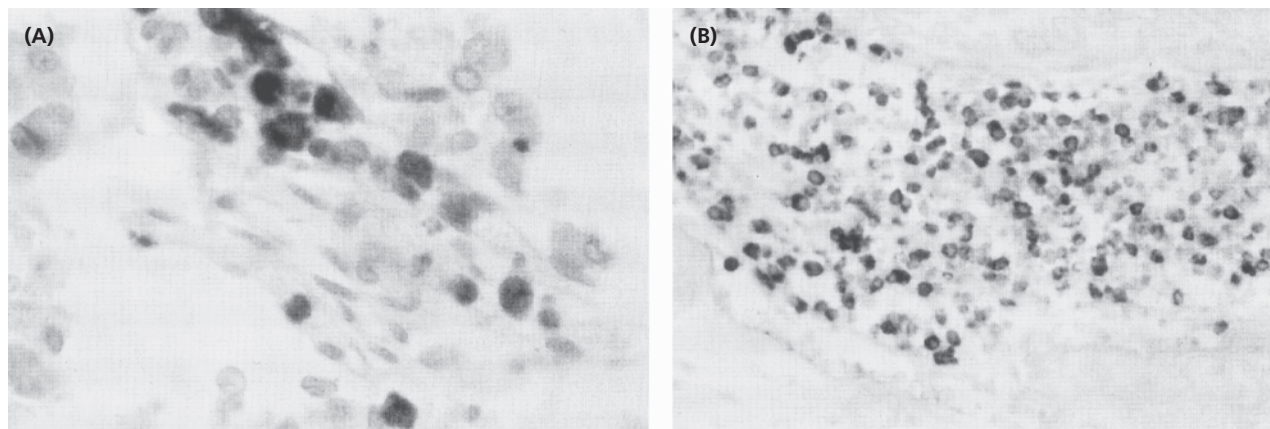


**Figure 25-3.** Appearance and subsequent expansion of neutrophils within the clavicular marrow cavity 12 to 15 weeks after conception. (A) At 12 weeks of gestation a small number of myeloperoxidase-positive cells are present in the developing marrow cavity. Many of these cells have the morphologic appearance of neutrophils with

segmented or band nuclei and they appear in discrete clusters within the marrow cavity. (Myeloperoxidase stain; original magnification,  $\times 400$ .) (B) At 15 weeks, clavicles have a marrow cavity that contains numerous myeloperoxidase-positive cells. (Original magnification,  $\times 200$ .) (Modified with permission from Slayton et al.<sup>4</sup>)

trimester progenitor cells with the capacity to generate neutrophils in vitro [colony-forming units-granulocyte-macrophage (CFU-GM)] are abundant in the early human fetal liver, marrow, and blood.<sup>12-17</sup> In rodents, the number of CFU-GMs per gram of body weight is far fewer in animals delivered prematurely than

in those delivered at term and is lower in term animals than in adults. The quantity of neutrophilic progenitor cells per gram of body weight in the developing human fetus has not been reported.<sup>18</sup> It is not clear whether, as in experimental animals, preterm human infants have a relatively small supply of granulocytic progenitors.

The concentration of CFU-GMs in the venous blood of adults is approximately 20 to 300/mL. In contrast, the concentration in term infants is approximately 2000/mL. Even higher concentrations are present in the blood of infants delivered prematurely. The high concentrations of CFU-GMs in fetal blood, however, do not necessarily indicate a large total body quantity of CFU-GMs. It is likely that a significant percentage of fetal CFU-GMs are in the circulation.<sup>16</sup>

When fetal CFU-GMs are cultured *in vitro* in the presence of recombinant G-CSF, they undergo maturation into colonies of neutrophils. Fetal CFU-GMs often mature clonally into larger colonies and contain more cells than do CFU-GMs obtained from the marrow of adults.<sup>19</sup> The physiologic role of G-CSF includes upregulation of neutrophil production. This appears to be the case for the fetus and neonate as well as for the adult. The low quantities of circulating and storage neutrophils in the midtrimester human fetus may be caused, in part, by low production of G-CSF. Supporting this hypothesis are observations of poor production of G-CSF by cells of human fetal origin.<sup>19,20</sup> Monocytes isolated from the blood of adults produce G-CSF when stimulated with a variety of inflammatory mediators, such as bacterial lipopolysaccharide or interleukin-1. In contrast, monocytes isolated from the umbilical cord blood of preterm infants and from the liver and marrow of aborted fetuses up to 24 weeks of gestation generate only small quantities (10 to 100 times less per cell) of G-CSF protein and mRNA after lipopolysaccharide or interleukin-1 stimulation.<sup>19,20</sup> Despite the poor capacity to generate G-CSF, it appears that G-CSF receptors on the surface of neutrophils of newborn infants are equal in number and affinity to those on adult neutrophils.

### Neonatal Neutropenia

Relatively few neutrophils are present in the human embryo and early fetus, and neutrophil production is a relatively minor component of hematopoiesis in the midtrimester fetus. On this basis, one might anticipate that neonates who are delivered extremely prematurely would be at high risk of serious bacterial infection. Indeed, of all the risk factors for neonatal infection analyzed in a national collaborative study<sup>21</sup> on neonatal infections, premature birth had the strongest correlation. One might also anticipate that premature neonates, delivered at the limits of viability (22 to 25 weeks of gestation) would have a high likelihood of developing neutropenia. This propensity is indeed seen. For instance, if neutropenia is defined as a blood neutrophil concentration  $<1000/\mu\text{L}$ , 40% of neonates weighing  $<1000\text{ g}$  at birth develop neutropenia at some time during their hospital stay.<sup>21</sup> Most such cases are detected on the first day after birth. Most are associated with maternal hypertension, or with a birthweight less than the 10th percentile for gestation, and most are relatively transient, with counts exceeding  $1000/\mu\text{L}$  within a few days (Fig 25-4). When neutropenia first appears after the third day of life, the underlying cause is usually obscure.<sup>21</sup> Neutropenia among extremely low birthweight neonates, although common, is viewed as problematic in only two situations—1) neutropenia

during an infectious illness among these patients is a very poor prognostic sign (see Chapter 30 for more information) and 2) neutropenia that is severe and prolonged is important to recognize, because it might indicate one of the syndromes or genetic abnormalities involving severe chronic neutropenia (see Chapter 14 for more information).

## Erythropoiesis

### Erythropoietin in the Fetus and Neonate

Production of erythrocytes requires a constant supply of amino acids, lipids, iron, specific vitamins, and trace nutrients.<sup>23</sup> Limitations in any of these can thwart production. However, the rate of erythrocyte production is regulated not by any of these substances, but rather by the concentration of erythropoietin. Erythropoietin is an 18.4-kD glycoprotein that binds to specific receptors on the surface of erythroid precursors and various other cells and supports their clonal maturation.<sup>23,24</sup> In the human fetus, erythropoietin is produced by a surprising variety of cells, including cells of monocyte-macrophage origin in the liver.<sup>25,26</sup> The absence of erythropoietin or its receptor, as has been produced in murine knock-out models, leads to profound anemia and fetal death on approximately embryonic day 13.<sup>27,28</sup> Postnatally, erythropoietin is produced almost exclusively by peritubular cells of the kidney, with a small amount produced by liver cells and neuronal and glial cells in the central nervous system. Although some erythropoietin production occurs in the fetal kidney, it is minimal. Anephric fetuses have normal serum erythropoietin concentrations and hematocrits.<sup>29</sup> The factors that regulate the switch of erythropoietin production from the liver to the kidney are not known. However, some investigators have suggested that among preterm neonates this switch does not occur until term gestation or close to it; this physiologic delay is responsible for the relatively low concentrations of circulating erythropoietin in the common hyporegenerative anemia known as anemia of prematurity.<sup>30</sup>

It has become clear that the actions of erythropoietin in the human fetus are widespread. Erythropoietin receptors have been documented on the surface of cells within a surprising variety of nonhematopoietic fetal tissues, including intestinal villi, endothelium, mesangium, smooth muscle, placenta, and neurons.<sup>25,26,31,32</sup> Erythropoietin has an antiapoptotic effect on several of these cell types *in vitro*. Erythropoietin also occurs in relatively high concentration in human amniotic fluid, colostrum, and milk—fluids that are swallowed in large amounts by the fetus and neonate.<sup>33</sup> A healthy midtrimester human fetus may swallow 200 to 300 mL of amniotic fluid per kilogram of body weight per day. With an amniotic fluid erythropoietin concentration of 200 mU/mL, the fetus would swallow approximately 60,000 mU/kg a day—an amount that if given systemically to a neonate would have a marked erythropoietic effect. Erythropoietin receptors are present along the villous border of the fetal and neonatal intestine.<sup>25,33</sup> It appears that

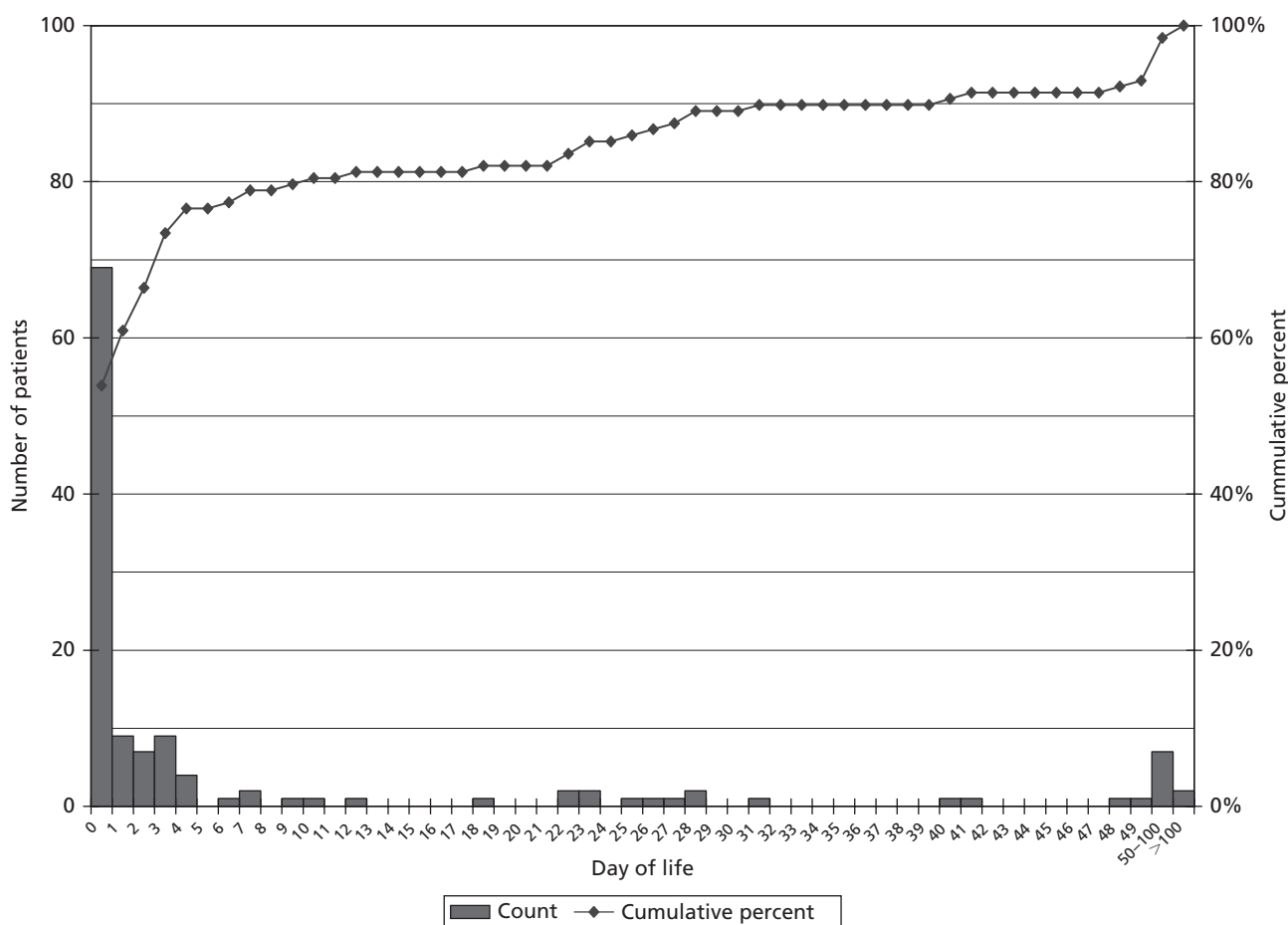


**Figure 25-4.** Day of life neutropenia (blood neutrophils  $<1000/\mu\text{L}$ ) was first detected among 338 neutropenia neonates with birthweights  $<1000$  g. (Used with permission from Christensen et al.<sup>21</sup>)

erythropoietin in amniotic fluid, colostrum, and human milk is not absorbed into the circulation and has no systemic effect. Rather, it acts locally in the intestine as a trophic and antiapoptotic factor. Erythropoietin in amniotic fluid, colostrum, and milk tends to resist digestive conditions present in the fetal and neonatal gastrointestinal tract.<sup>33,34</sup>

Erythropoietin is present, in concentrations of 20 to 50 mU/mL, in the spinal fluid of neonates. Markedly greater spinal fluid concentrations of erythropoietin are seen after perinatal asphyxia, and this erythropoietin appears to be derived within the brain as opposed to crossing the blood-brain barrier.<sup>35,36</sup> Further evidence that erythropoietin does not cross the blood-brain barrier in neonatal humans includes the observation that neonates treated with recombinant erythropoietin do not have elevated erythropoietin concentrations in their spinal fluid.<sup>36</sup> The spinal fluid of adults generally has an erythropoietin concentration lower than the lower limit of detectability.<sup>35,36</sup>

The biologic roles of erythropoietin and erythropoietin receptors in the fetal and neonatal intestinal tract and central nervous system are not known. One potential function for erythropoietin in the fetal brain is as a neuroprotectant.<sup>37</sup> This

postulate is supported by the observations that erythropoietin production increases in the brain during fetal hypoxia, and that recombinant erythropoietin protects neurons in tissue culture from hypoxemic damage and does so by diminishing apoptosis, which is analogous to the function it has in erythropoiesis.

### Fetal Erythroid Progenitors

Culture of marrow cells in tissue culture has added to the understanding of erythropoietic regulation. When marrow cells are placed in semisolid media culture systems for 5 to 7 days, the erythropoietin-sensitive precursors [colony-forming units–erythroid (CFU-E)] mature clonally into clusters containing 30 to 100 normoblasts.<sup>38</sup> Erythroid-specific progenitors that are less well differentiated than CFU-E, and hence more primitive cells, are called burst-forming units–erythroid (BFU-E). Twelve to 14 days after marrow cells are placed in semisolid culture systems, BFU-E have developed into large clusters of normoblasts, each containing 200 to more than 10,000 normoblasts. BFU-E from human fetuses respond in a slightly different manner than do BFU-E isolated from adults. Specifically, BFU-E of fetal origin generally develop into erythroid clones more rapidly and generally

develop substantially more normoblasts than do BFU-E of adult origin.<sup>39,40</sup> Also, BFU-E from adult marrow require a combination of erythropoietin plus another factor, such as interleukin-3 or GM-CSF, to mature clonally, whereas many fetal BFU-E mature in the presence of erythropoietin alone.<sup>39,40</sup>

### Embryonic, Fetal, and Adult Hemoglobins

Tissues must receive a constant supply of oxygen. The development of oxygen-carrying proteins increases the ability of blood to transport oxygen. The binding of oxygen and its dissociation from hemoglobin are accomplished without expenditure of metabolic energy.<sup>41</sup> Hemoglobin consists of iron-containing heme groups and globin, a protein moiety. An interaction between heme, globin, and 2,3-diphosphoglycerate (also called 2,3-bisphosphoglycerate) gives hemoglobin its unique properties in the reversible transport of oxygen. Hemoglobin is a tetrameric molecule composed of two pairs of polypeptide chains, each encoded by a different family of genes; the  $\alpha$ -like globin genes on chromosome 16 and the  $\beta$ -like globin genes on chromosome 11. The main hemoglobin of normal adults (HbA) is made up of one pair each of  $\alpha$  and  $\beta$  chains ( $\alpha_2\beta_2$ ). Six distinct hemoglobins can be detected within the erythrocytes of the human embryo, fetus, child, and adult—Gower-1, Gower-2, Portland, fetal hemoglobin (HbF), and the adult hemoglobins HbA and HbA<sub>2</sub>. The time of appearance and quantitative relations among the hemoglobins are determined by complex developmental processes that are not well defined.

Human embryos have the slowly migrating hemoglobins Gower-1, Gower-2, and Portland. The  $\zeta$  chains of Portland and Gower-1 hemoglobins are structurally similar to  $\alpha$  chains. Both Gower hemoglobins contain a unique polypeptide chain, the  $\epsilon$  chain. Gower-1 hemoglobin has the structure  $\zeta_2\epsilon_2$  and Gower-2,  $\alpha_2\epsilon_2$ . Portland hemoglobin has the structure  $\zeta_2\eta_2$ . Four to 8 weeks after conception, the Gower hemoglobins predominate, but by 12 to 14 weeks, they are no longer detected.

Fetal hemoglobin contains  $\gamma$  chains in place of the  $\beta$  chains of HbA and are represented as  $\alpha_2\gamma_2$ . The resistance of HbF to denaturation by strong alkali usually is used in its quantitation. After the eighth postconceptional week, HbF is the predominant hemoglobin. At 24 weeks of gestation, it constitutes 90% of total hemoglobin. Thereafter, a gradual decline in HbF occurs, so that at birth the average is 70% of the total. Synthesis of HbF decreases rapidly postnatally, and by 6 to 12 months of age only a trace is present. Fetal hemoglobin is heterogeneous because of two types of  $\gamma$  chains, synthesis of which is directed by two sets of genes. The chains differ at position 136 in the presence of either a glycine (G $\gamma$ ) or an alanine (A $\gamma$ ) residue. In the neonate, the relative proportion of G $\gamma$  to A $\gamma$  chain is 3:1.

Trace quantities of HbA can be detected in embryos. Thus, it is possible to make an early prenatal diagnosis of major  $\beta$ -chain hemoglobinopathy. Prenatal diagnosis is based on techniques used to examine the rates of synthesis of  $\beta$  chains or the structure of newly synthesized  $\beta$  chains or on molecular techniques from sampling the chorionic villus tissue or amniotic fluid. Gene

deletion disorders, such as  $\alpha$ -thalassemia, can be detected with the same methods.

At 24 weeks of gestation, approximately 5% to 10% of hemoglobin in a fetus is HbA. A steady increase follows, so that at term, the proportion of HbA averages 30%. By 1 year of age, the normal adult hemoglobin pattern appears. The minor adult hemoglobin component (HbA<sub>2</sub>) contains  $\delta$  chains and has the structure  $\alpha_2\delta_2$ . It is seen only when significant amounts of HbA are present. At birth, less than 1% of HbA<sub>2</sub> is present, but by 12 months, the normal level of 2% to 4% is attained. Throughout life the normal ratio of HbA to HbA<sub>2</sub> is approximately 30:1.

In the fetus and neonate, the rates of synthesis of  $\gamma$  and  $\beta$  chains and the amounts of HbA and HbF are inversely related. This has been attributed to a switch mechanism, but the developmental processes that direct the switch from predominantly  $\gamma$ -chain synthesis in utero to predominantly  $\beta$ -chain synthesis after birth are unclear. Primitive erythrocyte progenitors undergoing clonal maturation in culture (BFU-E) predominantly generate HbF. This may be the basis for the increased levels of HbF that occur in anemia with severe erythropoietic stress. Alternative explanations involve more basic genetic regulators in the DNA sequences that flank the hemoglobin gene complexes.

Because hemoglobins containing  $\epsilon$  chains are normally present only very early in intrauterine life, they are largely of theoretic interest. Small amounts of the Gower hemoglobins have been detectable in a few neonates with trisomy 13. Increased levels of Portland hemoglobin have been found in cord blood of stillborn infants with homozygous  $\alpha$ -thalassemia.

The normal adult level of HbA<sub>2</sub> (2.4% to 3.4%) is seldom altered. Levels of HbA<sub>2</sub> exceeding 3.4% are found in most persons with the  $\beta$ -thalassemia trait and in those with megaloblastic anemia secondary to vitamin B12 and folic acid deficiency. Decreased HbA<sub>2</sub> levels are found in those with iron deficiency anemia and thalassemia major.<sup>42</sup>

Erythrocytes of the midtrimester fetus and preterm infant are extremely large and laden with hemoglobin (Fig 25-5). At 22 to 23 weeks of gestation, the mean corpuscular volume (MCV) can be 135 fL or more, compared with a value of  $88 \pm 8$  fL in adults. Similarly, at 22 to 23 weeks of gestation, the mean corpuscular hemoglobin (MCH) can be over 45 pg, compared with  $29 \pm 2$  pg in adults.<sup>43</sup> Even at term, the MCV and MCH are strikingly higher than the upper limit of normal among healthy adults. However, throughout gestation the mean cell hemoglobin concentration is essentially the same as in adults.<sup>43</sup> Presumably, these very large and hemoglobin-laden erythrocytes are advantageous to the early fetus, although it is not clear what those advantages are. Perhaps the very high content of hemoglobin carried by these large cells somehow constitutes a fetal advantage. Whether large erythrocytes are also advantageous for prematurely delivered neonates ex utero is not known. Many preterm neonates have large phlebotomy losses in the first week of NICU care and are transfused with much smaller erythrocytes from adult donors. The consequences of this change in erythrocyte size are not known.

Thrombopoiesis

Platelets were described in the early part of the 19th century, but it was not until 1882 that their role in hemostasis was recognized. The origin of platelets from megakaryocytes was reported in 1906,<sup>44</sup> and since then the study of mechanisms responsible for platelet production has focused on megakaryocytes. The megakaryocyte compartment in the marrow consists of two pools of cells.<sup>45</sup> One is composed of cells that are morphologically unrecognizable but are committed to the megakaryocyte lineage. These cells, the megakaryocyte progenitors, retain a high proliferative capacity and ultimately determine the number of megakaryocytes in the marrow. The other pool consists of nondividing cells that are morphologically recognizable as megakaryocytes and undergo endoreduplication or

endomitosis, a process in which the ploidy of the cell increases without cellular division.

Fetal and Neonatal Megakaryocyte Progenitors

Progenitor cells committed to the megakaryocyte lineage can be identified by two methods: a culture system, in which they are identified by their ability to form megakaryocyte colonies, and immunologic staining, which allows characterization according to the specific antigens expressed on their membranes. With these methods, two different megakaryocyte progenitor cells have been identified; the burst-forming unit–megakaryocyte (BFU-MK), which is a more primitive megakaryocyte progenitor,<sup>46,47</sup> and a later progenitor known as the colony-forming unit–megakaryocyte (CFU-MK).<sup>45,48</sup> In culture, CFU-MK-derived colonies are smaller (3 to 50 cells per colony) and primarily unifocal

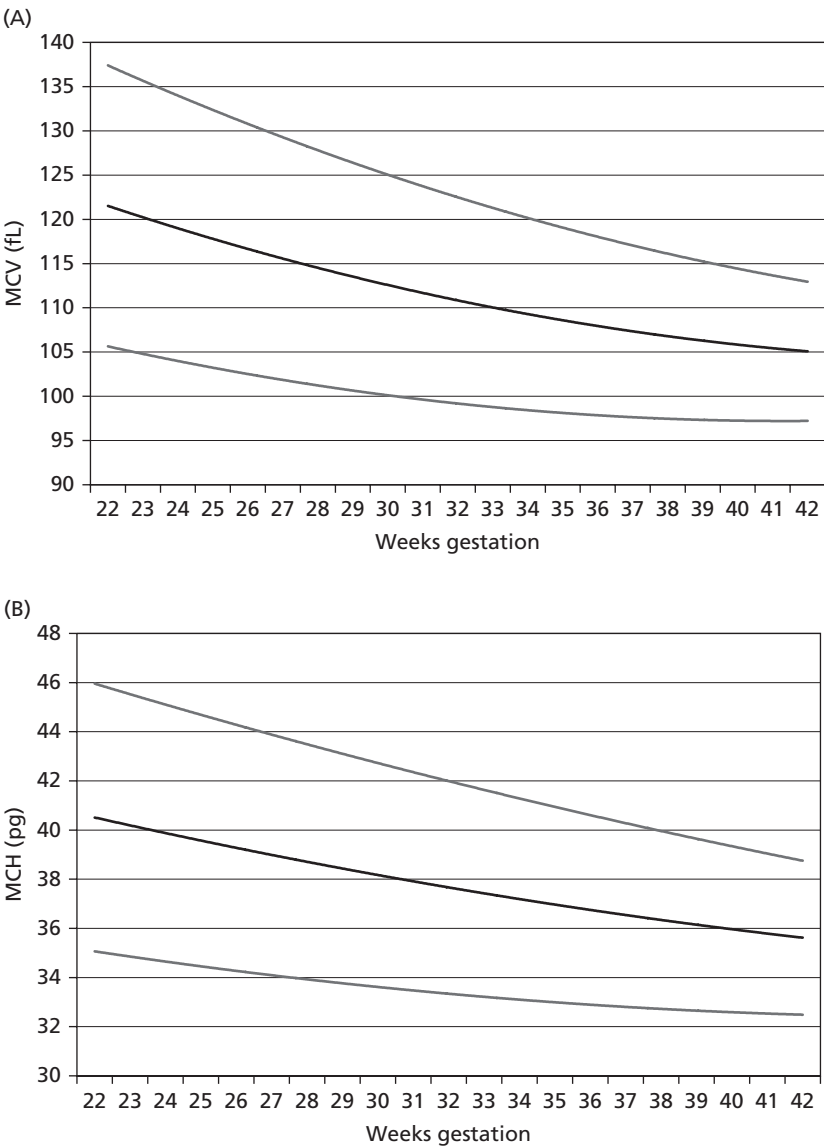


**Figure 25-5.** The expected range of values for (A) mean corpuscular volume (MCV) and for (B) mean corpuscular hemoglobin (MCH) for neonates of 22 weeks through 42 weeks of gestation, as measured on the first day after birth. The upper and lower boundaries incorporate 95% of the measured values. (Used with permission from Christensen et al.<sup>43</sup>)

(Fig 25-6), whereas BFU-MK-derived colonies are larger (>50 cells per colony) and have multiple foci of development. When immunologically phenotyped, CFU-MKs express CD34 and HLA-DR, whereas BFU-MKs express CD34 but not HLA-DR.

There are no differences between fetal and adult megakaryocyte progenitors in regard to the expression profile of CD34 and HLA-DR.<sup>49</sup> However, fetal BFU-MK-derived colonies are significantly larger than adult BFU-MK-derived colonies and are usually composed of only one or two foci of development. Adult BFU-MK-derived colonies are typically multifocal.<sup>49</sup> In addition, a unique megakaryocyte progenitor present in fetal marrow has an unusually high proliferative potential and gives

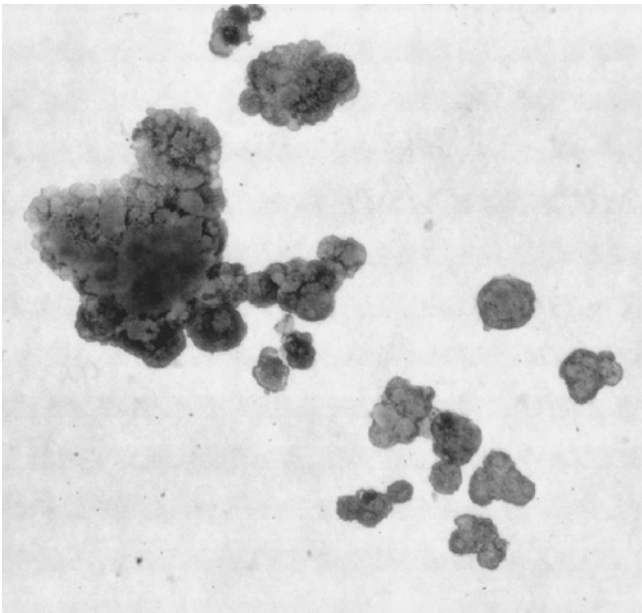


**Figure 25-6.** Photomicrograph showing a megakaryocyte colony derived from a megakaryocyte progenitor (CFU-MK) from the marrow of a thrombocytopenic preterm neonate. (Original magnification,  $\times 400$ ). Light-density mononuclear cells isolated from the marrow were cultured in collagen-based serum-free media with 50 ng/mL of recombinant human thrombopoietin. After fixation, the colonies were stained with a monoclonal antibody against glycoprotein IIb/IIIa to allow accurate identification of megakaryocytes.

rise to very large unifocal colonies (>300 cells).<sup>50</sup> This cell, not found in adult marrow cultures, may represent a more primitive megakaryocyte progenitor. The development of miniaturized assay systems to study megakaryocyte progenitors has made it possible to study these cells in the peripheral blood of neonates. Using these techniques, it has been shown that preterm neonates (24 to 36 weeks) have higher circulating concentrations of all megakaryocyte progenitors than do term neonates.<sup>51,52</sup>

**Fetal and Neonatal Megakaryocytes**

Unlike their progenitors, megakaryocytes have no proliferative abilities but undergo a complex maturational process. Through this process, they evolve from small, mononuclear cells to very large, polyploid cells easily recognized in the marrow as mature megakaryocytes.<sup>53,54</sup> The modal ploidy is 16N in normal adult marrow.<sup>55</sup>

In the human fetus, megakaryocytes are first detected in the circulatory system at 8 weeks of gestation, and the first platelets appear at 5 weeks.<sup>56</sup> Compared with the megakaryocytes of adults, fetal megakaryocytes are significantly smaller at all stages of maturation. Their ploidy distribution is also shifted to the left, with a higher proportion of immature megakaryocytes.<sup>57–59</sup> The modal ploidy in the marrow of near-term fetuses is 8N.<sup>60</sup>

Umbilical cord blood has higher concentrations of circulating megakaryocytes than adult blood. As in the fetus, cord blood megakaryocytes from term infants are considerably smaller than adult circulating megakaryocytes,<sup>61</sup> although they are otherwise phenotypically mature megakaryocytes. “Adult-size” megakaryocytes appear by 2 years of age, but the evolution of the process of megakaryocytopoiesis in the first year of life is poorly understood because of the lack of normal marrow specimens. Because cord-blood-derived megakaryocytes have been shown to generate fewer platelets than adult-derived megakaryocytes,<sup>62</sup> it has been postulated that the normal platelet counts of fetuses and neonates are maintained by the increased proliferative potential of the fetal/neonatal megakaryocyte progenitors (Table 25-3).<sup>63</sup>

The process of platelet production and release is one of the less well understood steps of thrombopoiesis. However, recent work has substantially enhanced our understanding of this process, and has painted a graphic picture of how megakaryocytes—in their

**Table 25-3.** Differences in Megakaryocytopoiesis Between Neonates and Adults

|                                     | Adults                                                                        | Neonates                                                                                                  |
|-------------------------------------|-------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------|
| Thrombopoietin (TPO) concentrations | Very high in hyporegenerative thrombocytopenia                                | Not as high in thrombocytopenic neonates (mostly small for gestational age) as in thrombocytopenic adults |
| Megakaryocyte progenitors           | Sparse in the blood<br>Give rise to small colonies<br>Less sensitive to TPO   | Abundant in the blood<br>Give rise to large colonies<br>More sensitive to TPO                             |
| Megakaryocytes                      | Large<br>High ploidy levels                                                   | Small<br>Low ploidy levels                                                                                |
| Effects of recombinant TPO          | Stimulates megakaryocyte proliferation<br>Stimulates megakaryocyte maturation | Stimulates megakaryocyte proliferation<br>Inhibits megakaryocyte maturation                               |

Used with permission from Sola-Visner.<sup>63</sup>



final hours—convert their cytoplasm into branched proplatelet extensions that elongate based on a process of microtubule sliding and polymerization.<sup>64</sup> Organelles and platelet-specific granules are delivered to the nascent platelets (at the tips of each proplatelet extension) by a mechanism involving both traveling of the organelles along the microtubules and movement of the linked microtubules relative to each other.<sup>65</sup> The role of blood-flow-induced shear stress on platelet release has also been evidenced in a recent study using intravital microscopy to observe in-vivo platelet release in intact murine marrow.<sup>66</sup> In this study, marrow megakaryocytes were observed to extend dynamic proplatelet-like protrusions into microvessels. These intravascular extensions were then sheared from their transendothelial stems by flowing blood, resulting in the appearance of proplatelets in peripheral blood.

### Thrombopoietin and Thrombopoietin Mimetics

Thrombopoietin (TPO), the main physiologic regulator of platelet production, was first isolated in 1994.<sup>67</sup> The gene that encodes thrombopoietin has been localized to the long arm of human chromosome 3.<sup>68</sup> Thrombopoietin mRNA is expressed primarily in the liver, and to a lesser extent in other tissues, including kidney and marrow stromal cells.<sup>69</sup> In vitro, TPO acts as a potent stimulator of all stages of megakaryocyte growth and development, except platelet release.<sup>70</sup> It also plays roles in platelet activation, mostly by priming the platelets to the effects of other agonists, and in release of platelets from megakaryocytes.<sup>71</sup> Thrombopoietin and TPO receptor knock-out mice models have been generated.<sup>72,73</sup> These mice have megakaryocyte and blood platelet concentrations of only 10% to 15% those of control mice. These studies confirmed that TPO is the primary regulator of platelet production, but also proved that alternative pathways exist for megakaryocytopoiesis.

Much attention has also been directed to the effects of TPO on other hematologic cell types. In vitro, TPO alone stimulates the proliferation and survival of erythroid, myeloid, and multipotential progenitors.<sup>74</sup> Thrombopoietin also enhances erythropoietin-induced erythroid burst formation, an effect mediated by its ability to inhibit apoptosis of erythroid progenitors.<sup>75</sup> Further studies have shown that TPO acts on early hematopoietic progenitors (including hematopoietic stem cells), thus disclosing an important role for this cytokine in hematopoiesis in general, in addition to its megakaryopoietic functions.<sup>76,77</sup> These findings have significant clinical implications for patients with mutations in the TPO receptor that render it unresponsive to TPO. These patients, such as those with congenital amegakaryocytic thrombocytopenia, frequently progress to aplastic anemia.

Results of several animal studies have confirmed the role of TPO as a potent stimulator of platelet production. The recombinant human full-length TPO molecule (rTPO) and a recombinant human polypeptide that contains the receptor-binding N-terminal domain of TPO (rHuMGDF) were the subjects of several studies. When injected into normal animals, rTPO and rHuMGDF induced marked thrombocytosis.<sup>78,79</sup>

Administered to animals exposed to myelosuppressive chemotherapy, rHuMGDF not only ameliorated the associated thrombocytopenia but also accelerated red cell and neutrophil recovery.<sup>80,81</sup>

Several Phase I and II trials in human subjects also demonstrated the efficacy of rTPO and rHuMGDF as stimulators of platelet production in adults without thrombocytopenia<sup>82</sup> and in patients with chemotherapy-induced thrombocytopenia.<sup>83,84</sup> However, the appearance of thrombocytopenia and ultimately aplastic anemia secondary to the generation of cross-reactive anti-TPO in a small number of patients receiving rHuMGDF<sup>85</sup> led to the discontinuation of all clinical trials involving any of the forms of rTPO. As an alternative, much interest has been directed recently to the development of TPO-mimetic molecules. These are small molecules that have no sequence homology to TPO, but bind to the TPO receptor and have biologically comparable effects. The lack of homology represents a significant advantage over recombinant forms of TPO, because it should preclude the development of cross-reactive neutralizing antibodies against endogenous TPO. At least five different TPO receptor agonists have been described, and at least two of them have been studied in human subjects: AMG 531, an engineered peptibody composed of a recombinant protein carrier FC domain linked to multiple c-mpl-binding domains,<sup>86,87</sup> and Eltrombopag (SB-497115), an oral, nonpeptide TPO receptor agonist.<sup>88</sup> Both compounds showed very promising results in patients with refractory immune thrombocytopenic purpura, and had a favorable safety profile.

### Thrombopoietin in the Fetus and Neonate

Little is known about the role of TPO or the theoretical benefits of administration of rTPO or TPO-mimetic compounds to neonates. Recombinant TPO certainly supports the growth of megakaryocyte colonies from the blood or marrow of neonates (Fig 25-6). In fact, marrow progenitors from neonates are more sensitive to rTPO than progenitors from adults in vitro (Fig 25-7).<sup>89</sup> Similarly, newborn rhesus monkeys are highly sensitive to rTPO in vivo (Fig 25-8) and respond to lower doses (per kilogram body weight) than those required in adult rhesus monkeys to achieve a similar effect.<sup>90</sup> Megakaryocyte progenitors from preterm neonates with or without thrombocytopenia also seem to be more sensitive to rTPO than progenitors from term neonates.<sup>91</sup> However, there are substantial qualitative differences in the response of neonatal and adult megakaryocytes to TPO, as demonstrated by a recent study evaluating the maturation of megakaryocytes cultured in either in TPO alone or in adult marrow stromal conditioned media.<sup>92</sup> In that study, adult megakaryocytes reached the highest ploidy levels when cultured in serum-free medium with maximal concentrations of rTPO, while neonatal megakaryocytes reached their highest ploidy when cultured in marrow stromal cell conditioned media *in the absence* of rTPO. In fact, the addition of supraphysiologic concentrations of this cytokine ( $\geq 0.1$  ng/mL) inhibited the maturation of neonatal megakaryocytes (Fig 25-9). Whether rTPO or

**Figure 25-7.** (A) Dose response to recombinant thrombopoietin of megakaryocyte progenitors obtained from the marrow of neonates with thrombocytopenia (T), neonates without thrombocytopenia (NT), and healthy adults. The marrow obtained from neonates generated approximately three times more colonies (per  $10^5$  low-density cells) than the marrow obtained from adults. (B) In a percentage of maximal colony count vs recombinant thrombopoietin concentration curve, the curves for the neonates with thrombocytopenia and those without thrombocytopenia reached a plateau at 10 ng/mL, compared with 50 ng/mL for the adults. This indicates that megakaryocyte progenitors from neonates are more sensitive to recombinant thrombopoietin in vitro than are their adult counterparts. (Used with permission from Sola et al.<sup>89</sup>)

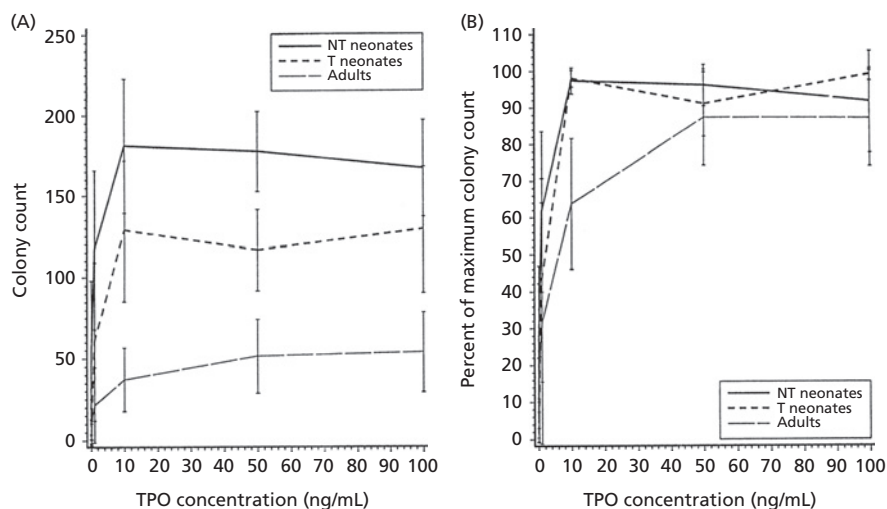


**Figure 25-8.** Dose-response to polyethylene glycol (PEG)-rHuMGDF (a truncated form of recombinant thrombopoietin) of the blood platelet concentration in newborn rhesus monkeys, demonstrating the sensitivity of megakaryocyte progenitors of neonates to recombinant thrombopoietin in vivo. The monkeys received daily subcutaneous injections of placebo or PEG-rHuMGDF at doses of 0.25, 1.00, or 2.50  $\mu$ g/kg/day for 7 days. Each line represents the average platelet counts of the two monkeys in each treatment group sampled on study Days 2, 4, and 6 and then twice weekly until Day 28. The peripheral platelet count increased on Day 6 of treatment, peaked on Day 11, and returned to baseline by Day 23. The two higher doses generated similar increases in platelet count. (Used with permission from Sola et al.<sup>90</sup>)

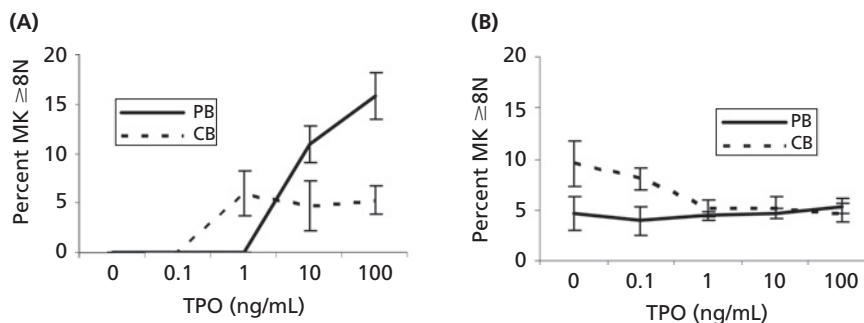
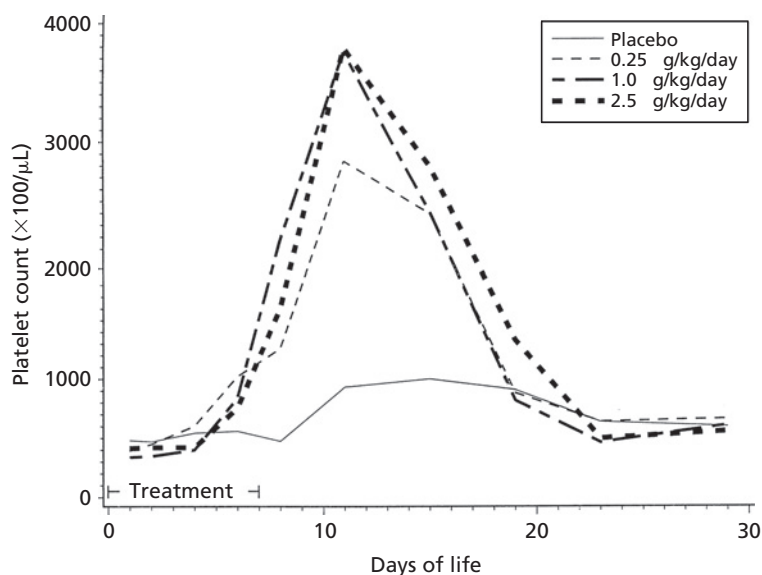


**Figure 25-9.** The percentage of megakaryocytes with ploidy levels  $\geq 8N$  in peripheral blood (PB)- and cord blood (CB)-derived megakaryocyte cultures differed depending on media source and recombinant thrombopoietin (rTPO) concentration. PB- and CB-CD34+ cells were cultured for 14 days in serum-free unconditioned media (A) and in adult marrow stromal conditioned media (B), with varying rTPO concentrations. PB-derived megakaryocytes (solid lines) cultured in unconditioned

media (A) exhibited a rTPO dose-dependent increase in ploidy levels, an effect inhibited by the presence of conditioned media (B). CB-derived megakaryocytes (dashed lines) reached highest ploidy levels when cultured in conditioned media with no rTPO, and effect that was reversed by rTPO concentrations  $\geq 1$  ng/mL (B). Data shown represent the means and standard error of the mean (SEM) of four separate experiments. (Used with permission from Pastos et al.<sup>92</sup>)



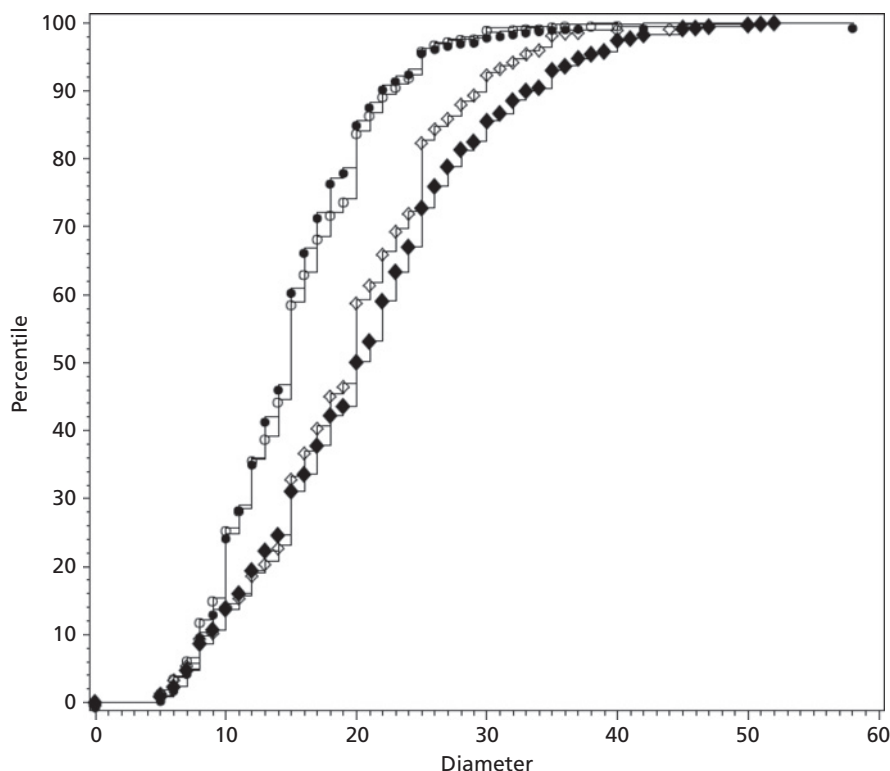


**Figure 25-10.** Cumulative distribution plots of megakaryocyte diameters in the marrow of thrombocytopenic neonates (dark circles), nonthrombocytopenic neonates (clear circles), thrombocytopenic adults (dark diamonds), and nonthrombocytopenic adults (clear diamonds). The megakaryocyte diameters are displayed on the X-axis, and their cumulative distribution on the Y-axis. Both adult curves are shifted to the right compared to the neonatal curves, indicating the predominance of larger megakaryocytes in these samples. Furthermore, the curve for the thrombocytopenic adults is also shifted to the right compared to that for the nonthrombocytopenic adults, indicating a higher percentage of large megakaryocytes. In contrast, the curves of thrombocytopenic and nonthrombocytopenic neonates are overlapping, indicating no change in the megakaryocyte size distribution in this cohort of thrombocytopenic neonates compared to controls. (Used with permission from Sola-Visner et al.<sup>96</sup>)

any of the TPO-mimetic peptides will be clinically useful as an alternative to platelet transfusions in the care of neonates with thrombocytopenia remains to be determined. However, it is clear that there are substantial developmental differences in megakaryocyte regulation, which will have to be taken into account when considering both the possible efficacy and the potential toxicity of thrombopoietic growth factors in this population.

### Neonatal Thrombocytopenia

When a fetus is delivered prematurely, the thrombopoietic system can be taxed by new demands. For instance, endothelial damage from infection or from the presence of intravascular catheters can accelerate platelet usage well beyond that normally experienced by the fetus. Moreover, disorders that tend to shorten gestation, such as pregnancy-induced hypertension and placental insufficiency, appear to have suppressive effects on fetal platelet production.<sup>93</sup> Because of these and other issues, thrombocytopenia is a very common problem in the neonatal intensive care unit, manifested in 20% to 30% of all NICU patients, and in 75% or more of the extremely low birthweight population.<sup>94</sup> Recent work has suggested that when fetuses or preterm neonates require an increase in platelet production, as a compensation for accelerated platelet usage or destruction, they might face developmental limitations in their ability to upregulate thrombopoiesis. Specifically, TPO concentrations in small-for-gestational-age neonates with hyporegenerative thrombocytopenia are less elevated than those reported in adults

with hyporegenerative thrombocytopenia, suggesting that—at least in this group of neonates—lower than expected TPO concentrations might contribute to the thrombocytopenia.<sup>91,95</sup> Furthermore, thrombocytopenic neonates in general do not seem to increase the size of their megakaryocytes as compared to their nonthrombocytopenic counterparts, thus blunting one of the mechanisms by which adults increase platelet production in response to platelet demand<sup>96</sup> (Fig 25-10). The mechanisms underlying the small size of neonatal megakaryocytes are not clearly understood, but recent studies suggest that they involve a combination of cell-intrinsic factors and factors in the fetal/neonatal microenvironment. Indeed, as demonstrated by stem cell transplant experiments, the adult environment is more conducive to megakaryocyte maturation than the fetal environment, but cell-intrinsic factors (such as the response to TPO) nevertheless limit the ultimate size and ploidy that neonatal megakaryocytes can achieve.<sup>97,98</sup>

### Summary

Neutropenia, anemia, and thrombocytopenia are relatively common problems in the NICU. These forms of cytopenia stem from multiple etiologic factors, and they involve many different kinetic mechanisms. However, all types of cytopenia in the NICU are more prevalent among those of shortest gestation and smallest birthweight. It is proposed that this relationship is

the result of gestational age-linked developmental differences in hematopoietic regulation. Transfusion is currently the main therapy available for NICU patients who have severe and prolonged cytopenia (see Chapter 30 for more information). As more is learned about human fetal hematopoiesis, perhaps alternatives to transfusion will emerge. To a certain extent, this process has already begun, because treatments such as recombinant erythropoietin and administration of recombinant G-CSF have been used in neonatology. However, much remains to be accomplished, such as more precise definitions of which neonates with cytopenias will benefit from treatment with recombinant hematopoietic growth factors, better methods for dosing and monitoring such treatments, and more precise means of identifying their risks and benefits.

## Disclaimer

The authors have disclosed no conflicts of interest.

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Because of physiologic hypervolemia in a healthy pregnant woman, a certain amount of blood loss after the birth of a child is considered normal. Postpartum hemorrhage (PPH) up to 1000 mL in a healthy mother, therefore, does not lead to disturbances in hemodynamics. PPH of more than 1000 mL in 24 hours occurs in 5% of deliveries. In women with anemia or poor increase in blood volume during pregnancy (eg, in preeclampsia), PPH less than 1000 mL can be hazardous to the mother. Blood loss in pregnancy, with consequences in oxygen-carrying capacity, does lead to additional danger for the fetus.

A worldwide survey shows huge variation in the lifetime risk of maternal mortality—from one in six in developing countries with a low human development index (HDI) to one in 30,000 in Northern European countries.<sup>1</sup> Obstetric hemorrhage and preeclampsia together account for 50% of maternal deaths in low HDI as well as high HDI countries.<sup>1,2</sup> Pregnancy complications and management differ in various parts of the world because of differences in blood groups, genetic and environmentally acquired diseases, socioeconomic status, and logistic and cultural factors.

## Blood Loss in Pregnancy

### Obstetric Causes

Delivery poses the highest risk for blood loss during pregnancy. If administration of at least 4 Red Blood Cell (RBC) units is used as a surrogate definition for major obstetric hemorrhage, then a national survey on severe maternal morbidity in the Netherlands has identified major obstetric hemorrhage in 4.5 per 1000 births.<sup>3</sup> Of a total of 358,874 births during the study period from August 2004 until August 2006, overall severe maternal morbidity occurred in 2552 women (7.1 per 1000 births). Of these

women, 1606 needed 4 or more RBC units, indicating that 70% of severe pregnancy complications are associated with severe hemorrhage. Data on the cause of bleeding were available for 1590 of the 1606 women. Table 26-1 shows the most frequent etiologies of the obstetric hemorrhages. In 1480 women (93%), blood loss occurred during the postpartum period. Antepartum hemorrhage occurred in 135 women (8.5%) and major bleeding in early pregnancy occurred in 51 women (3.2%). These

**Table 26-1.** Primary Diagnosis in Major Obstetric Hemorrhage Defined as the Need for at Least 4 Units of Blood<sup>3</sup>

| Timing                      | Diagnosis*                            | No. of Patients |
|-----------------------------|---------------------------------------|-----------------|
| Early pregnancy<br>(n = 51) | Ectopic pregnancy                     | 29 (56.9%)      |
|                             | Spontaneous abortion                  | 10 (19.6%)      |
|                             | Termination of pregnancy              | 10 (19.6%)      |
|                             | Miscellaneous†                        | 2 (3.9%)        |
| Antepartum<br>(n = 135)‡    | Abruptio placentae                    | 61 (45.5%)      |
|                             | Placenta previa                       | 54 (40.3%)      |
|                             | Miscellaneous§                        | 7 (5.2%)        |
|                             | Unknown diagnosis                     | 12 (9.0%)       |
| Postpartum<br>(n = 1480)*   | Retained placenta or placental rests  | 703 (47.8%)     |
|                             | Uterine atonia                        | 567 (38.5%)     |
|                             | Hemorrhage following cesarean section | 183 (12.4%)     |
|                             | Perineal tears/episiotomy             | 148 (10.1%)     |
|                             | Clotting disorders                    | 116 (7.9%)      |
|                             | Placenta accreta/increta/percreta     | 109 (7.4%)      |
|                             | Rupture of cervix                     | 58 (3.9%)       |
|                             | Uterine rupture                       | 44 (3.0%)       |
|                             | Uterine inversion                     | 13 (0.9%)       |
|                             | Miscellaneous                         | 65 (4.4%)       |
|                             | Unknown diagnosis                     | 10 (0.7%)       |

\*Up to three postpartum diagnoses could be coded.

†Molar pregnancy and placenta percreta.

‡In 76 cases both antepartum and postpartum diagnoses were coded.

§Rupture of uterine/ovarian artery, rupture of ovarian cyst, placenta percreta, vasa previa, retroplacental hematoma, rupture of uterine vein.

percentages total more than 100, because 76 women experienced both antepartum and postpartum bleeding. Because only those women in need of at least 4 RBC units were included in the survey, the most frequent causes of obstetric blood loss before birth (such as miscarriage and unexplained minor antepartum hemorrhage) are relatively underrepresented in these figures.

Assuming that the 239 women who received 10 or more units of blood would have died if they had not received those transfusions, the maternal mortality ratio (MMR) in the Netherlands would stand at 70 instead of the actual 12 per 100,000 live births. This underscores the impact of a functioning blood transfusion service on the reduction of maternal mortality. Lack of access to sufficient blood leads to a higher frequency of hysterectomy to stop the bleeding. Although hysterectomy may be a lifesaving procedure for some women, it adds to the risk of dying for others.

The MMR for Jehovah's Witnesses in the Netherlands was recently estimated to be 68 per 100,000 live births (van Roosmalen JJM, personal communication). This is strikingly similar to the likely MMR if blood had not been available for those women who received 10 or more RBC units.

Hematologic Diseases

Maternal diseases such as diabetes, renal failure, hemoglobinopathies, systemic lupus erythematosus, and antiphospholipid antibody syndrome (APAS) are not associated with primary bleeding. Rather, they are associated with increased maternal and fetal mortality resulting from thrombotic and vascular complications leading to preeclampsia, thromboembolism, and placental insufficiency. A few maternal diseases, such as maternal thrombocytopenia and (iatrogenic) coagulopathies, can pose primary bleeding problems during pregnancy and delivery; however, in the absence of obstetric complications, these problems rarely lead to maternal mortality.

Maternal Thrombocytopenia

A platelet count below 150,000/ $\mu$ L is a rather frequent finding in pregnancy. The possible causes are listed in Table 26-2.

Spurious thrombocytopenia caused by laboratory errors or EDTA agglutination is excluded by examination of a blood film or platelet counting in citrated blood.

Table 26-2. Causes of Thrombocytopenia in Pregnancy

|                                                                  |
|------------------------------------------------------------------|
| • Spurious thrombocytopenia                                      |
| • Gestational thrombocytopenia                                   |
| • Preeclampsia and HELLP                                         |
| • Thrombotic microangiopathies (HELLP, TTP, HUS)                 |
| • Disseminated intravascular coagulation                         |
| • Idiopathic/immune thrombocytopenic purpura                     |
| • Antiphospholipid antibody syndrome                             |
| • Congenital thrombocytopenia and/or platelet function disorders |

HELLP = hemolysis, elevated liver enzymes, and low platelet count; TTP = thrombotic thrombocytopenic purpura; HUS = hemolytic uremic syndrome.

Thrombocytopenia (platelet count between 80,000 and 150,000/ $\mu$ L) of unknown origin, often referred to as gestational thrombocytopenia (GT), occurs in approximately 6% of third trimesters.<sup>4</sup> Its cause and significance is often unknown. GT has been considered as subclinical immune thrombocytopenic purpura (ITP) or as a precursor of HELLP (hemolysis, elevated liver enzymes, low platelet count) syndrome.<sup>5,6</sup> GT is a diagnosis of exclusion. If, in uncomplicated pregnancy, the blood film is normal in an otherwise healthy female without a history of ITP or antiphospholipid antibodies, no diagnostic tests are needed. However, such women should be monitored carefully for aggravation of thrombocytopenia and symptoms of HELLP after delivery, because they may be at higher risk for postpartum HELLP syndrome.

HELLP syndrome belongs to the microangiopathic hemolytic anemias, also referred to as thrombotic microangiopathic anemias (TMAs), which can complicate pregnancy and puerperium.<sup>7,8</sup> The peak incidence has a relationship with the weeks of gestation (Fig 26-1), but there is considerable overlap with other syndromes (see below).

HELLP complicates 4% to 15% of cases of preeclampsia (hypertension and proteinuria). Its peak incidence is at 36 weeks, although in one-third of cases HELLP presents up to 7 days after a normal delivery not complicated by preeclampsia and in 10% it presents before the 27th week of gestation.<sup>9</sup> Typical symptoms include right-upper-quadrant abdominal pain, nausea, and vomiting. HELLP is not associated with overt disseminated intravascular coagulation (DIC), although in 70% the antithrombin level is decreased in contrast to thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (TTP/HUS).<sup>10</sup> Because of vasoconstriction in preeclampsia, severe bleeding rarely occurs. Severe neglected HELLP, often resulting from delayed delivery, leads to DIC. Other complications are pulmonary edema, acute renal failure, abruptio placentae, and liver hematoma. Maternal mortality is as high as 20% and neonatal death ranges from 10% to 60% depending on intervention with elective preterm deliveries, which reduce the risk of death to both mother and fetus. Mild thrombocytopenia and schistocytes are found in 50% of the neonates and resolve without treatment or transfusions. Because termination of pregnancy is the definitive treatment, elective birth should be considered. Severe preeclampsia and HELLP will subsequently resolve 1 to 10 days after termination of pregnancy. If recovery is delayed beyond 3 days or HELLP after delivery progresses to (multi)-organ failure, plasma exchange may support recovery.<sup>11</sup> The ranges of laboratory values that can be found in HELLP are shown in Table 26-3.

HUS typically presents from 2 days after delivery onwards (mean 26 days), although in 5% to 10% of cases antepartum HUS has been described. At presentation 50% of females have thrombocytopenia (platelet counts less than 100,000/ $\mu$ L).<sup>8</sup> Despite plasma exchange, approximately 25% of the patients experience persistent renal failure.

TTP results from an antibody-mediated reduction in the level of von Willebrand factor (vWF)-cleaving protease (ADAMTS13).

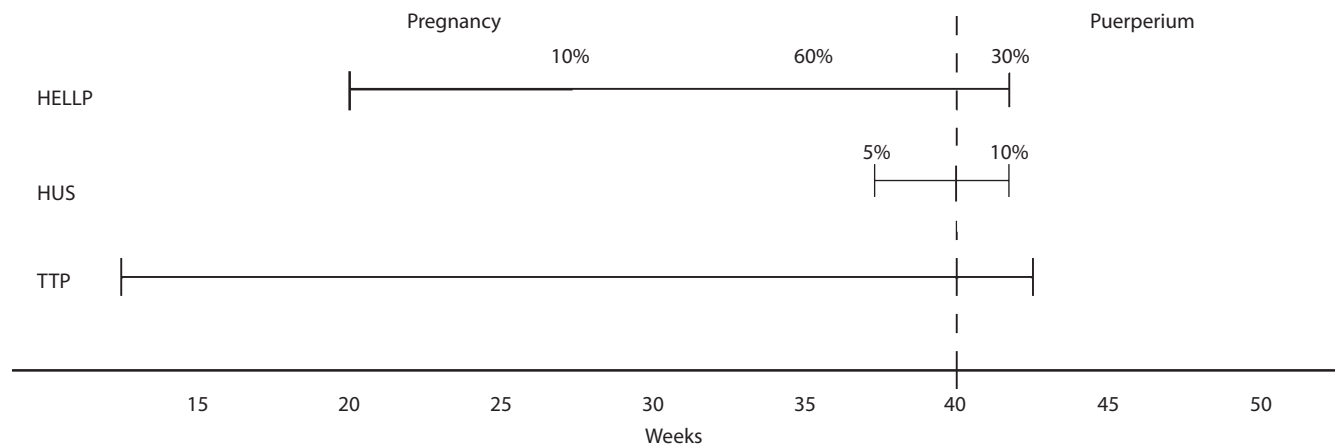


**Figure 26-1.** Pregnancy-associated microangiopathic thrombocytopenias. HELLP = hemolysis, elevated liver enzymes, and low platelet count; HUS = hemolytic uremic syndrome; TTP = thrombotic thrombocytopenic purpura.

**Table 26-3.** Laboratory Values in 442 Cases of HELLP<sup>7</sup>

| Test                              | Median | Range      |
|-----------------------------------|--------|------------|
| Platelets<br>(×1000/μL)           | 57.00  | 7-99       |
| Alanine aminotransferase<br>(U/L) | 249.00 | 70-6193    |
| Lactate dehydrogenase<br>(U/L)    | 853.00 | 564-23.584 |
| Bilirubin<br>(μmol/L)             | 26.00  | 8.6-436    |
| (mg/dL)                           | 1.53   | 0.51-25.65 |
| Creatinine<br>(μmol/L)            | 97.00  | 53-1414    |
| (mg/dL)                           | 1.10   | 0.6-16.1   |
| Urine acid<br>(μmol/L)            | 462.00 | 174-900    |
| (mg/dL)                           | 7.70   | 2.9-15     |

TTP is most often seen in the second and third trimesters and requires plasma exchange similar to treatment of TTP in non-obstetric situations. TTP has no relationship with preeclampsia and there is no need to terminate pregnancy. Patients with a history of TTP can have a relapse in pregnancy, but after remission pregnancy is not contraindicated, although close monitoring of the ADAMTS13 level is recommended.<sup>12</sup>

DIC is seen in far-advanced TMA syndromes, predominantly caused by primary obstetric or septic pathology, such as abruptio placentae and severe intrauterine infection. Between 20% and 40% of obstetric patients with critical illness have symptoms of DIC. In pregnancy and puerperium the use of a DIC score (based on decreased platelet count, increased D-dimers, decreased fibrinogen, and prolonged prothrombin time) is less reliable because D-dimers start to increase in the first trimester

and are always increased after the 35th week. Fibrin deposition in DIC not only contributes to multiple organ failure, but also (because of consumption coagulopathy with massive and ongoing activation of coagulation) results in depletion of platelets and coagulation factors with severe bleeding.

With an incidence of 6/100,000 new cases per year, ITP mostly affects young women.<sup>13,14</sup> Consequently, a history or current evidence of ITP is frequently encountered in pregnancy (estimated to be 1-5 cases per 10,000 pregnancies).<sup>15</sup> Often the mother has a history of ITP with or without a low platelet count, although first presentation of ITP or accidentally discovered ITP in pregnancy also occurs. During pregnancy platelets decrease with a nadir in the third trimester.<sup>16,17</sup> Antenatal treatment is given exclusively on maternal indication and aims to maintain the platelet count above 20,000 to 30,000/μL or higher in case of bleeding symptoms, using corticosteroids as first-line treatment.<sup>13,17</sup> Intravenous immunoglobulin (IVIG) is reserved as second-line treatment. IVIG is used to prepare for delivery, if necessary, combined with platelet transfusions. There is no demonstrated effect of maternal treatment with corticosteroids or IVIG on the fetal platelet count.<sup>18,19</sup> Perinatal mortality in ITP is 0.6%.<sup>20</sup> The risk for neonatal thrombocytopenia below 50,000/μL ranges from 8.9% to 14.7%.<sup>19-22</sup> Intracranial hemorrhage (ICH) or other severe bleeding is not increased in the fetus of a mother with ITP. The nadir of the neonatal platelet count and the highest risk for bleeding is 4 (range, 1-7) days after delivery.<sup>15-17</sup> In large series, ICH in the neonate is reported to be 0% to 1.5%.<sup>13,17,23</sup>

Most studies have reported only weak correlations between the fetal platelet count at birth and the maternal platelet counts, the presence of antibodies in the maternal serum, or a history of splenectomy.<sup>13,17</sup> In individual cases, the risk for fetal thrombocytopenia below 50,000/μL cannot be predicted from the maternal platelet count. However, in women who are refractory to corticosteroids and splenectomy and who cannot maintain a platelet count above 30,000/μL despite immunosuppressive treatment, the risk for bleeding and neonatal thrombocytopenia

is higher.<sup>16,17,24</sup> In asymptomatic women, severe fetal thrombocytopenia is rare and approaches the background rate of approximately 1% in term newborns. In approximately 70% of multigravidae, the absence of thrombocytopenia in a previous child predicts a similar good outcome in the next child.<sup>25</sup>

Antiphospholipid antibody syndrome is an acquired condition of hypercoagulability, often associated with systemic lupus erythematosus (SLE). APAS is characterized by arterial and venous thrombosis and gestational vascular complications leading to recurrent fetal loss, growth retardation, and prematurity. In patients with suspected clinical symptoms, antibodies against  $\beta$ 2-glycoprotein I, anticardiolipin autoantibodies, and/or prolongation of plasma clotting time (lupus anticoagulant) are present.<sup>26</sup> On the basis of randomized studies, women with recurrent abortion are treated with a combination of low dosages of aspirin and low-molecular-weight heparin.<sup>23,26</sup> There is no risk for fetal bleeding but the mother has a slight increased risk for bleeding during delivery.

Treatment of inherited disorders of hemostasis and platelet function disorders (such as von Willebrand disease, hemophilia, or Glanzmann's thrombasthenia) aims to avoid blood components when possible, at least during pregnancy, because of the risk of alloimmunization. Before and after delivery, blood transfusion may be unavoidable; this can be problematic if the patient is extensively immunized. Planned delivery and an individualized treatment plan are needed for these rare diseases.

### Chronic Maternal Anemia

Physiologic anemia is caused by plasma volume expansion and is maximal around the 25th week of pregnancy. The World Health Organization defines anemia in pregnancy as a hemoglobin level  $<6.8$  mmol/L (11 g/dL), which is most often caused by nutritional deficiencies. Below a hemoglobin level of 5.5 to 6.2 mmol/L (9–10 g/dL) growth retardation and prematurity are reported, although it is not known whether these are related to the underlying disease or the lower hemoglobin level itself.<sup>27–29</sup>

For chronic hemolytic diseases, such as thalassemia and paroxysmal nocturnal hemoglobinuria, hypertransfusion is applied to maintain hemoglobin levels above 10 g/dL in order to depress hemolysis of autologous red cells.<sup>30</sup> Randomized studies have involved only pregnant patients with sickle cell disease and have showed that maintenance of a hematocrit above 33% with less than 35% sickled cells resulted in no better pregnancy outcome, although painful crises were significantly reduced.<sup>31</sup>

### Assessment of Amount of Blood Loss

Visual estimation is frequently used in routine practice to assess the amount of blood loss. However, this technique is inaccurate and leads to significant underestimates of blood loss.

In a nicely researched experiment using pictorial guidelines to facilitate visual estimation (Fig 26-2), all professionals involved in obstetric care underestimated blood loss.<sup>32</sup> In fact, obstetricians tended to underestimate blood loss to a higher degree than anesthetists, surgical nurses, and midwives. The guidelines used in this study (available at <http://www.bmfms.org.uk>) can be used

as an effective tool to train health-care professionals to improve their abilities to estimate blood loss.

Other methods of estimating blood loss include measuring actual blood loss with a weighing scale or measuring the peripartum hemoglobin change. Measurement with a scale, however, must be corrected for the amount of amniotic fluid and is cumbersome. Peripartum hemoglobin change has been shown to be unrelated to visually estimated blood loss.<sup>33</sup>

In other studies the need for additional therapeutic uterotonic drugs or blood transfusion is taken as an indication of the amount of blood loss.<sup>34</sup> This is not relevant for clinical practice, but is important for audit purposes.

## Maternal Transfusions

### Indications

The indications for maternal transfusion can be categorized in two groups—those occurring after delivery and those occurring during pregnancy or delivery.

### Transfusion Indications for Postpartum Bleeding

Forty percent of women lose more than 500 mL of blood after vaginal delivery and 30% lose more than 1000 mL after cesarean section. As discussed above, the blood loss is difficult to estimate.<sup>33</sup>

Although guidelines suggest a transfusion threshold at a hemoglobin concentration of 7.0 to 8.0 g/dL, concentrations of 5.0 g/dL or more are usually well tolerated if isovolemia is maintained. In a study of healthy individuals, Weiskopf et al<sup>35</sup> found that acute isovolumetric reduction of hemoglobin concentration to 5.0 g/dL does not appear to cause inadequate tissue oxygenation. In healthy women with a median hemoglobin level of 10.5 g/dL (range, 7–15 g/dL) 3 to 6 weeks after delivery, no relationship between hemoglobin level and the quality of life was observed.<sup>36</sup> Studies of the effects of blood transfusion after PPH on quality of life are not available thus far.

Transfusion is required only for symptomatic anemia and should not be given solely on the basis of the hemoglobin level. Limited data exist on outcomes at concentrations below 5.0 g/dL. Two retrospective studies involving patients who declined blood transfusion, mostly Jehovah's Witnesses, found that morbidity and mortality rates were extremely high below this hemoglobin level.<sup>37,38</sup> However, survival has been reported at hemoglobin levels below 2.0 g/dL—even as low as 1.4 g/dL.<sup>39,40</sup>

Life-threatening hemorrhages after delivery are often associated with complex coagulopathies. Therefore, a management protocol must be in place.<sup>41–43</sup> Recent protocols advocate that when massive bleeding in complex trauma is anticipated, the patient should be transfused earlier without waiting for consumption and dilutional coagulopathies. This has been shown to reduce total blood use and mortality.<sup>44,45</sup> This seems to hold for obstetric bleeding as well. Control of bleeding, early transfusion, and correction of coagulopathy must be carried out during the window of opportunity, which may be less than 2 hours, before



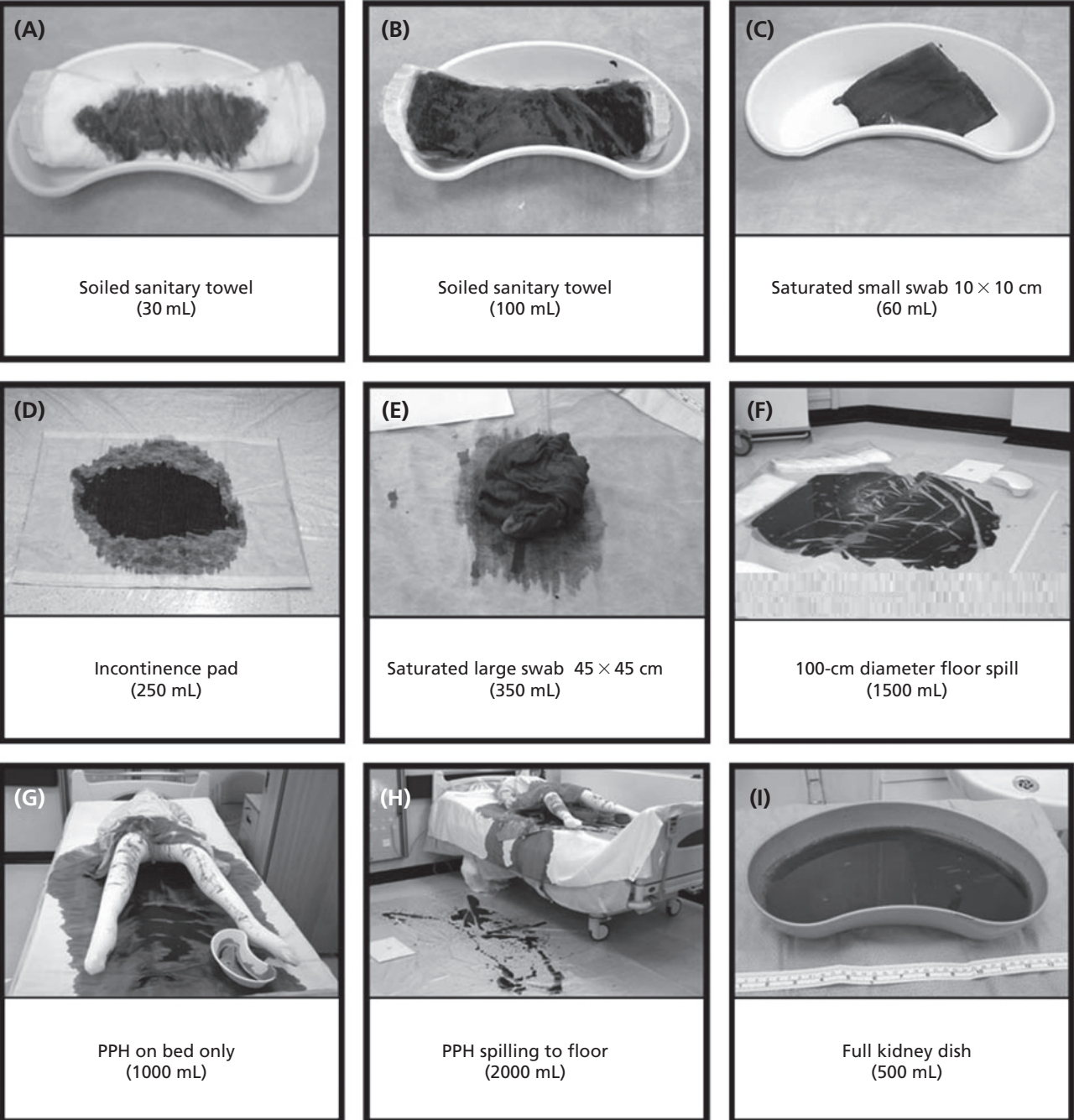


**Figure 26-2.** Pictorial guidelines to facilitate visual estimation of blood loss from obstetric hemorrhage.<sup>32</sup>

the vicious circle initiated by hypothermia, acidosis, and irreversible coagulopathy leads to death.

**Platelet Transfusions during Pregnancy and for Delivery**

During pregnancy, thrombocytopenias result from enhanced platelet phagocytosis in ITP or consumption in TMA, both disorders in which platelet transfusions are reserved for therapeutic indications. Reduced marrow platelet production

is rare unless a hematologic or oncologic condition is present. Whatever the cause of thrombocytopenia, most guidelines recommend a platelet count above 50,000/ $\mu$ L for delivery and 80,000/ $\mu$ L for cesarean section, allowing all forms of anesthesia.<sup>13</sup> Because of different pathophysiology, an individual treatment plan for every pregnant patient with thrombocytopenia is required. Except in cases of TTP, platelet transfusions are not contraindicated in TMA, ITP, or DIC. However, because platelet survival is

shortened, other supportive treatment intended to improve the cause of thrombocytopenia is required. (See Chapter 23.)

## Blood Products Used and Special Precautions Industrialized Countries with a High HDI

### Red Cell Transfusions

Pregnancy and transfusions both stimulate red cell antibody formation. A survey of the transfused population in developed countries shows that women have three or four times more alloantibodies than men and children.<sup>46,47</sup> Females with a history of transfusion and pregnancy must be screened early in a new pregnancy to identify alloantibodies as a potential cause for hemolytic disease of the fetus and newborn (HDFN) or availability of blood in case an indication for maternal transfusion should arise. In particular, antibodies against c and K1 antigens can cause HDFN as severe as Rh(D) HDFN.<sup>48</sup> In cases of HDFN caused by c or K1 antigens, more than 40% of the women had received prior transfusions.<sup>49</sup> Transfusions of Rh- and K-compatible components to females of childbearing potential can reduce non-D HDFN. Most national guidelines recommend the use of K-negative donors for women  $\leq 45$  years of age.

### Platelet Transfusions

Platelet transfusions can contain minimal numbers of erythrocytes, sufficient to immunize against the D antigen. If D-positive platelets are given to D-negative females of childbearing potential, prophylaxis with 375 IU intravenous Rh Immune Globulin (RhIG) is indicated.<sup>50</sup>

With increasing numbers of pregnancies, females produce leukocyte antibodies against paternal antigens, which may impair platelet recovery after transfusions from randomly selected donors.<sup>51,52</sup> HLA antibodies must be excluded if a need for platelet transfusion during delivery is anticipated. Because pregnancy stimulates immune anti-A and B, major ABO-incompatible platelet transfusions also show reduced recovery.<sup>53</sup>

### Immunoglobulin

Immunoglobulin preparations are used during pregnancy for immunomodulatory purposes to prevent formation of anti-D, increase the maternal platelet count in ITP, and reduce severe fetal bleeding in fetal and neonatal thrombocytopenia (FNAIT). To prevent Rh HDFN, D-negative women are given RhIG between the 28th and 34th weeks of gestation and also within 72 hours after delivery of a D-positive infant.<sup>54</sup> This reduces Rh HDFN to pregnancies of less than 1% of D-negative women. Nevertheless, Rh HDFN is still the most frequent and severe type of HDFN. The residual immunization results from medical, laboratory, and clerical errors, or an inadequate dose of RhIG after larger fetomaternal hemorrhages during delivery, after trauma, and after obstetric interventions.<sup>55,56</sup>

### General Aspects of Transfusion Products

Blood components can transmit cytomegalovirus (CMV) and parvovirus B19—infections that may harm the fetus. Blood

**Table 26-4.** Risk Factors for Postpartum Hemorrhage

- 
- Overdistended uterus (multiple pregnancy, large fetus, and polyhydramnios)
  - Prolonged labor
  - Induction of augmentation of labor
  - Postpartum hemorrhage in previous pregnancy
  - Chorioamnionitis
  - High parity
  - Coagulation disorders (placental abruption, syndrome of hemolysis, elevated liver enzymes, and low platelet count)
- 

components selected to reduce CMV and parvovirus B19 risk should be administered. Filtration of cellular blood components virtually abolishes CMV transmission. Pooled plasma products (eg, IVIG) must contain less than  $10^4$  copies of parvovirus B19, a load that is neutralized by antibodies present in the product. However, this does not protect against parvovirus B19 in individual cellular products.

### Indications and Practice

Prevention of PPH can be achieved by active management of the third stage of labor.<sup>34</sup> Although active management combines the use of oxytocic drugs, early cord clamping, and active efforts to deliver the placenta, actual practice differs, sometimes including selective radiologic embolization of uterine vessels, and sometimes consisting only of oxytocic drugs.<sup>57</sup>

Postpartum hemorrhage may occur in any woman in labor. Risk factors, however, will help the clinician anticipate the problem (Table 26-4).

The indications for transfusion are guided in the Netherlands by the 4-5-6 Flexinorm.<sup>58</sup> In the United States, guidelines are promulgated by the American Society of Anesthesiologists (ASA). When the hemoglobin levels is  $<4$  mmol/L (hematocrit 20%) in ASA category I patients (healthy persons) with acute blood loss, transfusion is indicated. In ASA category II patients (mild systemic disease without disability) and category III patients (systemic disease and serious disability), the threshold for transfusion lies at a hemoglobin level  $<5$  mmol/L (hematocrit 25%). In ASA category IV patients (constant life-threatening condition) the transfusion threshold is a hemoglobin level  $<6$  mmol/L (hematocrit 30%). In massive hemorrhage, Fresh Frozen Plasma is used to counteract clotting disorders. Preeclampsia, placental abruption, and amniotic fluid embolism are the most important obstetric disorders that may be accompanied by clotting problems. A woman with preeclampsia is already in a hypovolemic condition before hemorrhage starts and this needs special attention. Urinary output is the best indicator of renal perfusion in a bleeding or preclampsic woman and should be at least 30 mL/hour.

### Countries with a Low HDI

In developing countries, blood transfusion services are generally not able to deliver the number of units needed to save the lives of

women with obstetric blood loss. In many places blood donation “on the spot” by relatives after the adverse event has taken place is the only available option. Transmission of hepatitis and human immunodeficiency virus (HIV) is a serious risk. In Tanzania, for instance, a National Blood Transfusion Service was developed in response to the HIV epidemic and a serious train crash in 2002. In 2006, 67,000 units of blood had been donated by volunteer donors. These nonremunerated donors have proven to be safer donors than relatives. However, there are not enough safe donors, and patients remain dependent on relatives as important sources for blood. It is tragic that these relatives have high rates of hepatitis and HIV infections.

Because anemia resulting from malaria, dietary deficiencies (iron and folic acid), sickle cell disease, and infectious disease is rampant in low HDI countries, a relatively small amount of blood loss is often disastrous. Thus, the World Health Organization defines PPH as a blood loss of >500 mL, while in many high HDI countries the threshold is >1000 mL.

Anemia should be treated rigorously during pregnancy in order to reduce the consequences of PPH.<sup>59</sup> The use of bednets, nutritional supplements, and antihelmintics; presumptive treatment of malaria; folic acid and ferrous medication during antenatal care; and active management of the third stage of labor all help women to overcome the problems created by hemorrhage during childbirth. These strategies may reduce the need for blood transfusion. Physicians should remember that unnecessary blood transfusion in a setting of limited resources is not only a waste but also likely to increase exposure to transfusion-related risks and infections.<sup>60</sup>

Transfusion or injection of unsafe blood is responsible for 8 to 16 million hepatitis B virus infections, 2.3 to 4.7 million hepatitis C virus infections, and 80,000 to 160,000 HIV infections annually. At present, 80% of the world’s population has access to only 20% of the world’s safe blood supply.<sup>60</sup> A recent paper from Nigeria reports a blood transfusion rate of 25.2% during cesarean section, a high rate that demonstrates the risk of surgery when resources are not available.<sup>61</sup> A strategy that safely reduces the number of cesarean sections also reduces the need for blood transfusion.

## Fetal Transfusions

This section discusses aspects of fetal transfusion focusing on transfusion for red cell immunization and the management of FNAIT.

### Alloimmune Hemolytic Disease

The first fetal transfusion was performed in 1963 by Liley.<sup>62</sup> After amniography and fetography, red cells were transfused under X-ray guidance in the intraperitoneal cavity of the fetus. This method of treatment of fetal anemia is based on the fact that red cells are absorbed from the peritoneal cavity and enter the circulation. Limitations of this technique include less uptake of the red cells in cases of severe fetal hydrops. Reported survival rates at the time were approximately 50%.<sup>63</sup>

The advent of real-time ultrasound ushered in a new era of fetal management. Since the 1980s fetal transfusions have been performed with the use of ultrasound guidance—specifically, ultrasound-guided puncture of the umbilical vein.<sup>64</sup>

Intravascular transfusions have been performed in thousands of pregnancies worldwide. Although randomized trials comparing intraperitoneal and intravascular transfusions have not been performed, results of the latter technique, which is feasible from 16 weeks of gestation onwards, are widely considered as superior.

Fetal blood sampling (FBS) by cordocentesis or puncture of the intrahepatic umbilical vein is indicated when fetal anemia is suspected (by use of Doppler measurements of the fetal vessels<sup>65</sup>) or in cases of hydrops. Intrauterine transfusion is performed directly after the diagnosis of anemia is established by FBS. The most frequent indication for fetal blood transfusion is anemia caused by red cell alloimmunization of the mother (see Chapter 27). Other indications for fetal blood transfusion include: human parvovirus B19 infection,<sup>66,67</sup> severe fetomaternal hemorrhage,<sup>68</sup> placental chorioangiomas,<sup>69</sup> and homozygous  $\alpha$ -thalassemia.<sup>70</sup> Placental chorioangioma and thalassemia are very rare indications and are not discussed further.

Fetal blood sampling is also applied for the management of other conditions such as thrombocytopenia or severe tachyarrhythmia of the unborn.

### The Technique

The principle of fetal transfusion is to access the fetal circulation with a needle under real-time ultrasound guidance, aspirate blood for diagnosis, and deliver red cells, platelets, or drugs to the fetal circulation. The most frequently used site for transfusion is the umbilical vein when the placenta is inserted anteriorly. When the umbilical vein at the placental insertion cannot be reached safely, the fetus is punctured in the intrahepatic vein. The umbilical vein is preferred for several reasons. First, the vein has a larger diameter than the arteries. Second, because of the eccentric location of the vein in the cord, an overshoot or slip of the needle does not in general lead to a hematoma in the Wharton’s jelly, but to a leakage in the amniotic cavity. A puncture in the artery may lead to leakage in the Wharton’s jelly, inducing spasm of the artery and subsequent fetal bradycardia. Third, fetal transfusion in the vein allows the operating team to monitor the flow of the transfused blood sonographically during the procedure. In some centers intravascular transfusion is combined with an intraperitoneal approach.

The procedure is performed with a 20- or 22-gauge spinal needle. Premedication to the mother is used for relief of maternal anxiety and sedation of the fetus. Routine intravenous or intramuscular administration of muscle relaxants such as atracurium or pancuronium is advocated to achieve fetal paralysis and thus prevent needle displacement caused by fetal movements.<sup>71</sup>

### Donor Blood, Volume, and Rate of Transfusion

The blood selected for fetal transfusion is group O, RhD-negative and compatible with any maternal antibodies that are present.

Because intrauterine transfusions easily stimulate new maternal antibodies against fetal or donor red cell antigens, a freshly derived maternal blood sample should be crossmatched before every subsequent transfusion.<sup>72</sup>

The selected blood component should not carry the risk of transmitting CMV and parvovirus B19 or causing graft-vs-host disease (GVHD). To promote optimal survival, the red cells are stored as briefly as possible. Before transfusion, supernatant solutions containing potassium, ABO antibodies, and red cell preservation solutions are often removed and replaced by saline. A leukocyte-reduced red cell concentrate in 0.9% saline with a hematocrit of 80% irradiated with 25 Gy just before transfusion can correct extremely low hemoglobin values without causing or transmitting disease.

After access to the fetal circulation, 1 to 2 mL blood is aspirated and promptly examined for hemoglobin, hematocrit, and mean corpuscular volume. Some facilities prefer to determine these values immediately, using a cell counter in the operating room. The volume of blood transfused is calculated by using a formula in which pretransfusion fetal hematocrit, the estimated fetal placental blood volume, and the hematocrit of the donor blood are included.<sup>73</sup> The blood is transfused at a rate of 5 to 10 mL per minute.<sup>73</sup> During transfusion, the blood flow and the fetal heart rate are monitored by ultrasound and Doppler. The second transfusion is given 1 to 2 weeks after the first. Following subsequent transfusions, fetal erythropoiesis is depressed and the fetus is completely dependent on transfusions every 3 to 4 weeks. At the end of treatment, more than 70% of women with HDFN requiring intrauterine transfusions have antibodies to multiple red cell antigens.<sup>72</sup> Selection of donors compatible with the mother for clinically relevant antigens could reduce this alloimmunization. In approximately half of the cases, additional antibody formation cannot be circumvented because the antibodies are evoked by fetal red cells.<sup>72</sup>

### Outcome of Treatment

At Leiden University Medical Center, 593 ultrasound-guided intravascular transfusions have been performed in 210 fetuses between 1988 and 1999. The transfusions were given between 17 and 35 weeks of gestation and resulted in an overall survival rate of 86%.<sup>48</sup> In the period from 1999 to 2006 the survival rate improved to 95% (unpublished data). The improvement in the survival rate results from fewer cases presenting with severe irreversible hydrops, after the 1997 implementation of routine screening for red cell antibodies early in pregnancy.<sup>50</sup> There has also been a reduction of procedure-related complications. Follow-up of the infants thus far has revealed no adverse effects of the treatment.

### Fetal and Neonatal Alloimmune Thrombocytopenia

In 0.3% of newborns, thrombocytopenia with an immunologic origin is encountered. Fetal and neonatal alloimmune thrombocytopenia (also known as neonatal alloimmune thrombocytopenia) and autoimmune thrombocytopenia (also known as ITP or Morbus Werlhof disease) are immune-mediated thrombocytopenias. In cases with severe red cell alloimmunization,

thrombocytopenia (platelet count  $<50,000/\mu\text{L}$ ) in the fetus is found before the first intrauterine transfusion in 3% of the cases. In severely hydropic fetuses this percentage is 10% to 30%.<sup>74</sup>

In these cases the mechanism causing thrombocytopenia is not known. Because severe in-utero bleeding is not documented in ITP<sup>19</sup> and the risk of ICH in the neonate is estimated at 0% to 1.5%,<sup>13,17,23</sup> intrauterine platelet transfusions are not indicated; the risk of complications exceed the risk of bleeding.

Approximately 2% of pregnant women of European ancestry are negative for HPA-1a. Anti-HPA-1a is responsible for the majority (75-95%) of cases of FNAIT.<sup>75</sup> About 5% to 25% of FNAIT is caused by other HPA antibodies, most frequently anti-HPA-5b, anti-HPA-3a, and anti-HPA-1b. In 15% to 20% of FNAIT cases no antibodies can be detected. Antibodies against private antigens and new antigens causing FNAIT are still being discovered.<sup>76</sup> In platelet-alloimmunized pregnant women carrying a fetus that is positive for the offending antigen, the risk for thrombocytopenia can be as high as 85%. The incidence of ICH varies from 7% (in alloimmunized pregnancies and a sibling without ICH) to 85% (in alloimmunized pregnancies and a sibling with ICH).<sup>77</sup> It is assumed that most ICH occurs antenatally between 30 to 35 weeks of gestation.

For antenatal management, it is important to know whether the father is homozygous or heterozygous for the offending antigen. In case of heterozygosity of the father, genotyping of the fetus is indicated. Although genotyping is possible by using polymerase chain reaction on either chorionic villi or amniocytes, amniocentesis in the second trimester is preferable because of its lower risk of boosting antibodies. Currently, methods are being developed to assess the fetal HPA type from fetal DNA in maternal plasma. When the fetus is positive for the offending antigen, the pregnancy should be managed in, or in collaboration with, a specialized fetal care center.

Because HPA screening is not routine, FNAIT is recognized most often only after an affected sibling is identified. The antenatal management protocols in FNAIT are intended to prevent ICH. Unfortunately, monitoring HPA antibodies by titration and quantification has not accurately predicted the severity of fetal thrombocytopenia. However, recent studies indicate that it is helpful in predicting the severity of fetal thrombocytopenia.<sup>78-80</sup> In order to diagnose fetal thrombocytopenia as early as possible, various centers have designed protocols for routine FBS beginning as early as 20 weeks.<sup>81,82</sup> In cases of fetal thrombocytopenia and platelet transfusion, the procedure must be repeated frequently, given the fact that the half-life of transfused platelets is only 5 days. Although FBS is a reliable tool for direct monitoring of the fetal thrombocytopenia, the cumulative procedure-related risk for fetal loss is high and approximates 6% per pregnancy.<sup>83,84</sup> The cumulative risk for emergency delivery due to FBS is 13% to 17%.<sup>85,86</sup> Currently, most centers have adopted the use of weekly high-dose maternal IVIG. A few centers use corticosteroids as first-line treatment. Although corticosteroids do not enhance the effect of IVIG,<sup>85,86</sup> combinations of these options are also in use. Bussel et al<sup>87</sup> were the first who reported in 1988 that

weekly maternal IVIG (1.0 g/kg maternal weight) was effective at elevating the fetal platelet count. Later studies showed that not all fetuses showed a substantial increase in platelet count with this treatment. The reported response rate in the literature varies between 30% and 85%. In addition, observational studies have suggested that IVIG reduces the risk of ICH even in nonresponders to IVIG,<sup>86,88,89</sup> possibly by downregulation of endothelial activation by platelet antibodies that cross-react with endothelial epithelial cells.<sup>90</sup>

In the past decade there has been a gradual change in antenatal treatment of platelet alloimmunized pregnancies, from an invasive approach with repetitive FBS to a less invasive approach (treatment with IVIG and less FBS or platelet transfusion) to a completely noninvasive approach (IVIG only). At Leiden University Medical Center, treatment of 52 pregnancies (53 neonates) at risk for fetal ICH (5 with and 48 without a sibling with ICH) resulted in term live births without ICH in all neonates.<sup>89</sup> Long-term follow-up of the infants, treated antenatally with IVIG, showed no adverse effects.<sup>91</sup>

The mechanism of action of IVIG in FNAIT is still unclear. There are several possible explanations. First, in the maternal circulation the IVIG will dilute the HPA antibodies, resulting in a lower proportion of HPA antibodies among the IgG transferred via the Fc $\gamma$  receptors (Fc $\gamma$ Rs) in the placenta. Second, in the placenta, IVIG may block the placenta receptor and decrease the placental transmission of maternal antibodies including anti-HPA. Third, in the fetus, IVIG may block the Fc $\gamma$ Rs on the macrophages and prohibit the destruction of antibody-covered platelets. Recently, Ni et al<sup>92</sup> presented a murine model of FNAIT, measuring response to IVIG therapy. This model demonstrates that maternal IVIG administration has multiple effects on the amelioration of FNAIT, including decreased maternal platelet antibody, decreased fetal platelet clearance, reduced bleeding disorders, and increased fetal survival.<sup>92</sup>

In a cost-effectiveness analysis, Thung et al<sup>94</sup> compared the use of noninvasive empiric IVIG with FBS treatment. They concluded that noninvasive IVIG is a cost-effective strategy.<sup>93</sup>

In the antenatal management of FNAIT, it is important to realize that the majority of at-risk patients are those who have a sibling with a history of severe thrombocytopenia but without internal bleeding. The risk of ICH in this category of patients is low and approximates the risk of fetal loss with invasive management; therefore, a noninvasive approach is preferred. Considering the high cost of IVIG, and that most of the ICH in these patients occurs later in pregnancy, treatment of FNAIT pregnancies without a history of ICH may be started between 28 and 32 weeks. In pregnancies with a previous sibling with an ICH, IVIG should be started at 12 to 18 weeks. To evaluate the response, FBS can be performed at 24 to 28 weeks. When the anticipated risk of the procedure is high (eg, less experienced center or a posteriorly located cord insertion), IVIG should be continued until delivery without FBS. A recent study examined this less invasive approach in seven pregnancies with a prior sibling with ICH.<sup>88</sup> IVIG (1 g/kg/week) was administered from a median of 16 weeks (range, 16–29 weeks)

without initial or follow-up FBS. Although all neonates had a birth platelet count of less than 50,000/ $\mu$ L, none of them showed signs of internal or external bleeding. The neonates needed only one platelet transfusion and did well. The results demonstrate that the primary goal in the management of pregnancies at risk for FNAIT is the prevention of severe bleeding complications and not thrombocytopenia per se. The very rare case of a patient with a history of ICH very early in pregnancy might benefit from a more aggressive approach (eg, high-dose IVIG, followed by repeated FBS and, when necessary, rescue high-dose corticosteroids).

In FNAIT patients, safety precautions may lower the risk of hemorrhage during FBS. The Leiden University Medical Center protocol for FBS includes the following:

1. FBS in FNAIT pregnancies is performed by the most experienced members of the fetal medicine unit.
2. The use of a cell counter in the operating room enables platelet count and histogram to be obtained within 2 minutes.
3. Before each FBS, compatible platelets are made available, and infused during the procedure, if necessary, to achieve a platelet count of  $>300,000/\mu$ L.

Platelet transfusions for fetal indications are generally blood group O RhD-negative (HPA compatible in case of FNAIT) units that have a reduced risk of CMV and parvovirus B19 transmission. The intent is to increase the platelet count to normal levels, preferentially higher than 300,000/ $\mu$ L to achieve a fetal platelet count  $>20,000/\mu$ L for 7 to 10 days. The recovery in the fetal circulation is approximately 25%, because 50% of the platelets remain in the placental bed and 50% of the platelets in the child are pooled in the spleen. This implies that approximately 100,000 to 200,000/ $\mu$ L platelets (depending on gestational age-related fetoplacental volume) are concentrated in 20 to 50 mL for intrauterine transfusion after irradiation with 25 Gy to prevent GVHD.

### Fetal Transfusions for Other Indications

Parvovirus B19 infection during pregnancy may lead to an arrest in maturation of hematopoietic stem cells and thus anemia and thrombocytopenia. Parvovirus B19 is a frequent cause of nonimmune hydrops fetalis and 46% of hydropic fetuses have thrombocytopenia below 50,000/ $\mu$ L. The platelet count will further decrease after transfusion of red cells alone.<sup>94</sup> After serologic and DNA diagnosis of infection in the mother and elevated middle cerebral artery peak velocity at Doppler investigation, intrauterine transfusion is indicated. Usually one intrauterine blood transfusion is sufficient.<sup>67</sup> Because a low platelet count ( $<50,000/\mu$ L) may lead to bleeding complications from the FBS, platelets should be available for transfusion.

Massive fetomaternal (transplacental) hemorrhage is a rare, unexpected, and serious complication in pregnancy resulting in severe fetal anemia, hydrops, and death. Reduced fetal movement is the only cognizant sign for the pregnant woman. The diagnosis can be confirmed when a sinusoidal pattern is observed at cardiotocogram registration and/or when an increased flow in the middle cerebral artery is measured.<sup>95</sup> Fetal red cells in the

maternal circulation can be confirmed with the Kleihauer-Betke test.<sup>93</sup> In the preterm period, fetal red cell transfusion is a realistic option.

After the introduction of laser coagulation of placental anastomosis as treatment for severe twin-to-twin transfusion syndrome (TTTS), TAPS (twin anemia-polycythemia sequence) has been described as a new syndrome.<sup>96</sup> After laser coagulation, very small residual anastomosis may lead to chronic anemia and polycythemia in one of the twins.<sup>97</sup> Robyr et al<sup>98</sup> described 13 cases of TAPS (13% of their group of TTTS cases). In 12 of the 13 cases, between one and five fetal red cell transfusions were performed. Because expectant management can lead to a favorable outcome, further research in this field is needed.

## Conclusion

Postpartum hemorrhage is a serious risk factor in obstetrics. Protocols for both prevention and management should be available in all labor and delivery wards. In high HDI countries, most maternal mortality can be prevented using oxytocics, embolization of uterine vessels, and blood transfusion. Unfortunately, both control of bleeding and early transfusion are difficult to achieve in most low HDI countries. In these countries, blood transfusion services are generally unable to help women who need red cells. Blood donation “on the spot” is often the only available option, and bears a serious risk for transmission of infections such as hepatitis and HIV. The option of fetal transfusion, a widely used treatment of the unborn in developed countries, is not available in many other countries.

## Acknowledgment

We are grateful to Mrs. Ivanka Bekker for expert secretarial assistance in the preparation of this chapter.

## Disclaimer

The authors have disclosed no conflicts of interest.

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Hemolytic disease of the fetus and newborn (HDFN) is caused by increased destruction of red cells in the fetus and newborn. The disease is associated with immune-mediated destruction (allo- or autoantibodies), intrinsic red cell abnormalities (hemoglobinopathies, enzyme defects, and membrane defects), and acquired red cell defects (related to infections, see Chapter 26). This chapter focuses on fetomaternal alloimmune-mediated HDFN, and only briefly mentions important intrinsic abnormalities.

**The Changing Spectrum of HDFN****Status in Developed Countries**

During the past 30 to 40 years, the spectrum of HDFN has changed profoundly. In the late 1960s and early 1970s, HDFN was a common neonatal problem dominated by Rh(D) alloimmunization. Overt fetal or neonatal hemolysis, with marked anemia and hyperbilirubinemia in the neonate, were commonly observed symptoms. The neonates were often severely affected and unstable at birth, and required multiple exchange transfusions. HDFN was associated with considerable neonatal morbidity and mortality.

Introduction in the 1970s of routine postpartum prophylactic Rh Immune Globulin (RhIG or anti-D) to D-negative women (with D-positive infants) and after pregnancy termination or abortion, has changed the picture dramatically and reduced D alloimmunization by about 90%.<sup>1</sup> After introduction of additional antepartum prophylactic RhIG, the frequency of D alloimmunization is now well below 0.2%.<sup>1</sup> The introduction of prophylactic RhIG ranks as one of the great success stories of modern perinatal care and immunology.

Programs for antibody screening and ABO/Rh typing of pregnant women allows focus on fetuses and neonates at risk, and has paved the way for the vastly improved antepartum surveillance and therapy outlined in Chapter 26.

Finally, postpartum therapy has improved considerably.<sup>2</sup> Forty years ago, exchange transfusion and early versions of phototherapy were the only therapeutic alternatives for serious HDFN. Today modern phototherapy, if necessary combined with high-dose intravenous immunoglobulin (IVIG), has reduced the need for exchange transfusion to single figures even in large centers. This raises serious challenges in order to keep the necessary competence in the practical execution of exchange transfusion.<sup>2</sup> Alloantibody frequencies in populations of mostly European ancestry before and after introduction of postpartum prophylactic RhIG treatment are indicated in Table 27-1.

**Status in Developing Countries**

Programs for antibody screening and ABO/Rh typing of pregnant women and introduction of postpartum routine prophylactic RhIG treatment have posed both organizational and economic challenges in countries with a low human development index (HDI). Improved neonatal surveillance and therapy has been

**Table 27-1.** Red Cell Antibodies in 1967 and 1995<sup>3,4</sup>

| Antibody       | 1967 (Minnesota, 7 years) | 1995 (Sweden, 12 years) |
|----------------|---------------------------|-------------------------|
| D              | 1864 (63.1%)              | 159 (19.0%)             |
| E              | 80 (2.7%)                 | 5 (6.1%)                |
| C              | 448 (15.2%)               | 36 (4.3%)               |
| C <sup>w</sup> | 4 (0.14%)                 | 10 (1.2%)               |
| c              | 68 (2.3%)                 | 38 (4.5%)               |
| e              | 2 (0.07%)                 | 1 (0.1%)                |
| K1             | 93 (3.1%)                 | 48 (5.7%)               |
| Duffy          | 17 (0.6%)                 | 26 (3.1%)               |
| MNSs           | 45 (1.5%)                 | 35 (4.2%)               |
| Kidd           | 7 (0.2%)                  | 10 (1.2%)               |
| Lutheran       | —                         | 13 (1.6%)               |
| P1             | 27 (0.9%)                 | 48 (5.7%)               |
| Lewis          | 94 (3.2%)                 | 241 (28.8%)             |
| I              | 13 (0.4%)                 | —                       |
| Others         | 194 (6.6%)                | 120 (14.4%)             |
| Total          | 2,956                     | 836                     |
| Blood samples  | 43,000                    | 110,765                 |

introduced only to a limited degree, and antepartum treatment is often nonexistent. HDFN is, therefore, still a major cause of morbidity and mortality in these countries.

### Serologic and Clinical Features

Development of immune HDFN requires maternal IgG antibodies directed against paternal antigens on fetal red cells. Immunization can occur when fetal red cells pass through the placenta to the mother's circulation or the antigens were present in previous transfusions of the mother. Only maternal IgG antibodies can cross the placenta. The transport is an active process dependent upon interaction between maternal IgG and syncytiotrophoblast Fc receptors, and is most active in the third trimester.<sup>5</sup> IgG antibodies can be elicited by most blood group antigens (Tables 27-1 and 27-2, and Chapter 5). The immune response to peptide antigens (eg, D) typically requires T-cell help, and is dominated by IgG antibodies. Because the D antigen is extraordinarily immunogenic and induces high affinity anti-D IgG, it has been a major cause for HDFN in White and Black populations where 10% to 15% of people lack the D antigen. This is not the case in East Asia (particularly China), where the population is D-positive (see Chapter 7). In addition, IgG autoantibodies causing maternal hemolytic anemia can result in HDFN. The common occurrence of antibodies reacting with carbohydrate antigens of the ABO, Hh, Ii, Lewis, and P systems without prior exposure to allogeneic red cells is a result of the wide distribution of these antigens in nature. IgM

is generally the dominating antibody, but IgG anti-A, anti-B, and more rarely anti-H are also present. Because the I antigen and antigens of the Lewis and P systems are almost lacking on fetal cells, they are not associated with HDFN.<sup>7</sup> Table 27-2 summarizes antibody frequencies and severity of HDFN caused by relevant blood group antigens in populations of European and African ancestry.

The direct antiglobulin test (DAT) (see Chapter 5) is one of the cornerstones for the diagnosis of HDFN. It detects IgG antibodies on the surface of red cells, and the number of IgG molecules bound to the erythrocyte surface determines the strength of the reaction. Using the spin antiglobulin test, the detection limit is 100 to 150 IgG molecules per red cell; when over 1000 are bound, the DAT is strongly positive (all cells are agglutinated).<sup>8</sup> Transplacental passage of maternal IgG results in antibody coating of the fetal red cells. High alloantibody concentrations lead to a strong DAT, whereas low concentrations or binding affinity result in a weak or, by traditional methods, often negative DAT. A strong positive DAT is observed particularly with anti-D; however, the reaction is more variable with alloantibodies against other Rh antigens and antigens such as Kell, Duffy, Kidd, and MNSs, which are well expressed on fetal red cells.<sup>5</sup> Because ABO antigens are not fully developed on fetal red cells and A and B substances are expressed by most tissues, the DAT in ABO incompatibility is often negative. However, with very sensitive techniques (eg, automated analyzer with low-ionic-strength medium with enhancing agents) IgG can almost always be demonstrated on the red cells in ABO incompatibility.<sup>9</sup> As a basic test, the DAT has poor predictive value in identifying neonates requiring treatment. Only 23% of newborns found to be DAT positive on neonatal screening have been reported to develop hyperbilirubinemia requiring treatment, but when the DAT was strongly positive (generally because of anti-D) all required treatment.<sup>10</sup> It should also be borne in mind that after antepartum treatment of D-negative women with prophylactic RhIG, neonates may demonstrate a positive DAT without signs of hemolysis.<sup>11</sup>

In an affected fetus, HDFN is caused by the hypoxic effects of anemia, while in the newborn, HDFN is caused by toxic effects of bilirubin and anemia. Bilirubin crosses the placenta during pregnancy and is removed by the mother's liver. However, because of insufficient capacity or failure of the neonate's liver, plasma levels of unconjugated bilirubin can quickly become high enough to cause serious central nervous system damage in the newborn. The anemia causes increased fetal erythropoiesis, which in severe cases leads to erythroblastosis with widespread extramedullary hematopoiesis and many nucleated red cells (erythroblasts) in the circulation. The most severe state of erythroblastosis, hydrops fetalis, can lead to stillbirth and is characterized by severe anemia, massive edema, and hypoproteinemia.

In severe erythroblastosis, blood film examination will show massive presence of nucleated red cells, but only a few spherocytes due to the rapid destruction of the red cells (eg, serious Rh HDFN). In less severe HDFN, spherocytes will dominate the blood film, and only a few nucleated red cells are observed (eg, ABO HDFN). This emphasizes the importance of blood film examination in neonates with nonphysiologic jaundice.<sup>2</sup>

**Table 27-2.** Blood Group Antigens and Hemolytic Disease in the Neonate<sup>6</sup> in Populations of European and African Ancestry

| Antigen                           | Antibody Frequency (per 1000 pregnant women) | Severity of HDFN in Infants with the Antigen |          |        |
|-----------------------------------|----------------------------------------------|----------------------------------------------|----------|--------|
|                                   |                                              | None/Mild                                    | Moderate | Severe |
| A, B, AB                          | Not relevant                                 | Not HDFN, but antibody in 90%                | <10%     | <1%    |
| D                                 | 2.6                                          | 51%                                          | 30%      | 19%    |
| c, cE                             | 0.9                                          | 70%                                          | 23%      | 7%     |
| E                                 | 2.0                                          | Almost all                                   | -        | Rare   |
| C, Ce, C <sup>w</sup> , e         | 0.7                                          | 86%                                          | 14%      | Rare   |
| Le <sup>a</sup> , Le <sup>b</sup> | 3.0                                          | Not HDFN                                     |          |        |
| Kell (K1)                         | 3.2                                          | 30-50%                                       | 30-37%   | 13-38% |
| Fy <sup>a</sup>                   | 0.8                                          | 67-94%                                       | 16%      | 6-16%  |
| Fy <sup>b</sup>                   | Rare                                         | Rare cause of mild HDFN                      |          |        |
| Kidd (Jk <sup>a</sup> )           | 0.2                                          |                                              |          | Rare   |
| M                                 | 0.5                                          |                                              |          | Rare   |
| N                                 | 0.1                                          |                                              |          | Rare   |
| U                                 | Rare                                         |                                              |          | Rare   |
| N                                 | 0.03                                         | Not HDFN                                     |          |        |
| P (P1)                            | 0.03                                         | Not HDFN                                     |          |        |

Persistent or prolonged jaundice in neonates is a common feature of HDFN, and can be observed in virtually all conditions mentioned in this chapter. Achieving the correct diagnosis is important for the neonate and can have implications also for other family members.

## Hemolytic Disease Predicted by Antepartum Maternal Antibody Screening

Although the majority of cases of acute neonatal hyperbilirubinemia/hemolysis currently occur without antepartum warning in high HDI countries, there remain a number of neonates for whom HDFN is predicted by antepartum screening. Almost all blood group antibodies of IgG type can cause HDFN. However, the severity and frequency of HDFN varies, and anti-D still remains the most common cause of severe HDFN.<sup>2</sup> The most frequent alloantibodies are directed against: Rh antigens (anti-D, anti-E, anti-c, anti-C), Kell antigens (anti-K1), Duffy antigens (anti-Fy<sup>a</sup>), Kidd antigens (anti-Jk<sup>a</sup>), and MNSs antigens (Table 27-2). Anti-D, anti-c, anti-E, and anti-K are found in about 1 per 1000 pregnant women. Anti-K is mostly caused by transfusion, and the frequency of HDFN due to anti-K is much lower than that due to anti-D.<sup>5</sup> However, anti-Kell can cause severe fetal anemia because the antibody also inhibits fetal erythropoiesis.<sup>12</sup> The frequency and severity of HDFN caused by the other antibodies mentioned above is low (Table 27-2). Positive antepartum maternal antibody screening results in frequent antepartum monitoring and makes treatment with intrauterine transfusions possible (see Chapter 26).

Antepartum diagnosis and postpartum management of neonates affected by HDFN require close collaboration among obstetric, neonatal, and transfusion medical teams. Delivery is generally induced around 36 weeks of gestation. All neonates at risk should have cord blood taken for measurement of hemoglobin, DAT, and bilirubin (see Chapter 26). Because of the use of intrauterine transfusions, many neonates have normal hemoglobin at birth and develop only modest hyperbilirubinemia. In these neonates, the DAT demonstrates few antibody-covered red cells, and typing barely reveals the neonate's original blood type. However, all potentially affected neonates should remain in the hospital until hyperbilirubinemia and/or anemia is under control and appropriate follow-up is organized. Phototherapy, high-dose IVIG, and exchange transfusions are often not required. Red cell transfusions can be indicated when anemia develops during the first couple of weeks after birth. Severe anemia (hemoglobin <10 g/dL) at birth or rapidly increasing hyperbilirubinemia should be treated as detailed later in this chapter.

## Early-Onset/Rapidly Progressive Hemolytic Disease not Predicted by Maternal Antibody Screening

Today neonatal pediatricians in developed countries are often alerted to HDFN in a relatively full-term infant whose mother's

antepartum screening was negative. Within the first 48 hours of life such a newborn develops early-onset jaundice with a total bilirubin already in excess of 20 mg/dL (342  $\mu$ mol/L).<sup>2</sup> Because the problem in such cases has not been predicted by antepartum screening for maternal antibodies, therapy would not have been started. The most common causes of HDFN presenting in this way are as follows:

- ABO incompatibility.
- Hemoglobinopathies.
- Erythrocyte enzyme defects.
- Disorders of the red cell membrane.

The reader is referred to Chapter 29 for detailed information on hemoglobinopathies, erythrocyte enzyme defects, and disorders of the red cell membrane.

### ABO Incompatibility

ABO incompatibility is now the leading cause of HDFN in high HDI countries.<sup>10</sup> HDFN resulting from ABO incompatibility generally occurs only in offspring of women of blood group O because IgG anti-A and anti-B is far more common in group O than in group A or B mothers, and is confined to the 1% with antepartum high-titer IgG antibodies.<sup>13</sup> Unlike in non-ABO HDFN, the first ABO-incompatible infant is at risk for significant hemolysis, because ABO antibodies are developed before pregnancy. Minor degrees of red cell destruction are common, as shown by the increased incidence of neonatal jaundice and slightly lower hemoglobin values in ABO-incompatible infants compared to ABO-compatible infants.<sup>5</sup> With respect to ethnic groups, the prevalence of maternal-newborn ABO incompatibility ranges from 31% in those of European ancestry to 50% in Asians. Generally, ABO HDFN causes mild postpartum jaundice, which is detected 24 hours after delivery; in very rare cases, however, hemolysis is severe enough to cause hydrops.<sup>14</sup> The frequency of HDFN caused by anti-A is only about 1 in 150 births, and even lower for anti-B.<sup>2</sup> Higher frequencies that have been reported from Africa and Saudi Arabia are probably related to more potent anti-A and anti-B (caused by environmental factors) and a relatively strong expression of A and B antigens.<sup>5</sup>

Antepartum and postpartum serologic tests are poor predictors of ABO hemolytic disease. The results of the DAT in HDFN caused by ABO incompatibility are very different from those observed with HDFN caused by anti-D (not treated antepartum). In the latter, a positive DAT can be observed even in cases showing minor clinical symptoms. However, in ABO incompatibility only 20% to 40% of incompatible pairs demonstrate a positive reaction and significant hemolysis can be observed with a negative or only weakly a positive DAT. In infants with a positive DAT result, the test differentiates poorly between newborns with clinical hemolysis and unaffected newborns. Only about 10% of the DAT-positive, ABO-incompatible maternal-fetal pairs develop clinically significant HDFN. However, in infants with clinically significant hemolysis, >80% have a positive DAT.<sup>10,15</sup> Also, the clinical symptoms differ in that anti-A and anti-B HDFN result mainly in hyperbilirubinemia detected 24 hours after delivery

without the significant fetal or neonatal anemia observed in anti-D HDFN. This is explained by the few and unbranched A and B antigen sites on fetal red cells, allowing antibody-coated cells to remain in the circulation longer than in anti-D HDFN. Another important factor is that ABO antigens are expressed on virtually all cells in the body, whereas D antigen is restricted to red cells. Thus, anti-D is “focused” to red cells, while anti-A and anti-B are absorbed by tissues and neutralized by soluble A and B substances in plasma. As a reflection of this, the blood film in anti-A or anti-B HDFN shows a large number of spherocytes, with little or no increase in nucleated red cells. In anti-D HDFN there are few spherocytes but large numbers of nucleated red cells. In ethnic groups expressing strong (many and branched) A and B antigen sites, severe anemia, nucleated red cells, and significant antepartum morbidity (even with hydrops fetalis) can be observed.<sup>2</sup>

Management of ABO HDFN is usually successful with modern phototherapy alone. Close monitoring of affected neonates is important, as high-dose IVIG adjuvant therapy and occasionally even exchange transfusion can be required. The latter is particularly relevant in individuals from certain ethnic groups who express strong A and B sites.<sup>2</sup>

### Hemoglobinopathies

Except for  $\alpha$ -thalassemia major, hemoglobinopathies generally do not cause antepartum or neonatal problems. Some occasional structural mutations in  $\alpha$  and  $\gamma$  genes that give no symptoms in adults, however, can result in transient hemolytic anemia in neonates. Mutations in the  $\beta$  genes (eg, sickle cell disease or  $\beta$ -thalassemia) give no symptoms at birth because of the predominance of fetal hemoglobin.

$\alpha$ -thalassemia major occurs when all four  $\alpha$ -globin genes on the homologous chromosome 16 are deleted ( $\alpha^0$ ), and is predominantly seen in some Mediterranean island populations and in Southeast Asia. The lack of  $\alpha$ -globin production leads to the dominating production of fetal  $\gamma_4$  hemoglobin (hemoglobin Bart's), which has a very high oxygen affinity and is physiologically useless. Infants are usually stillborn between 28 and 40 weeks, demonstrating the typical picture of hydrops fetalis with pallor, generalized edema, and massive hepatosplenomegaly. The few that are live-born expire within the first hours after birth. Postpartum management has no impact on survival unless combined with carefully planned antepartum therapy, and has to be followed up with lifelong red cell transfusions or hematopoietic stem cell transplantation. Checking blood samples from the parents will show whether they are carriers of the  $\alpha^0$  gene (hypochromic, microcytic red cells). The diagnosis is confirmed by hemoglobin electrophoresis.

$\alpha$ - and  $\gamma$ -globin chain structural abnormalities are generally clinical silent, but can result in occasional neonatal hemolytic anemia due to formation of unstable  $\alpha\gamma$ -hemoglobin. When the fetal  $\gamma$ -globin is replaced by adult  $\beta$ -globin, the hemolytic anemia resolves. Diagnosis is confirmed by hemoglobin high-performance liquid chromatography. For further details and information see Chapter 29.

### Red Cell Enzyme Defects

The important enzyme defects in the neonatal period are glucose-6-phosphate dehydrogenase (G6PD) deficiency and pyruvate kinase deficiency. They usually present with early onset, often severe, hyperbilirubinemia. Blood smear is normal, and anemia rare. Family medical history and measurement of the relevant pretransfusion enzyme activities are of diagnostic importance.

G6PD deficiency is transmitted in an X-linked recessive form, and seen in all ethnic groups; however, its highest prevalence is in central Africa (20%) and the Mediterranean region (10%). It is not clear why only some neonates develop jaundice. However, because these patients can develop severe hyperbilirubinemia, close monitoring is important. Interaction with other risk factors for hyperbilirubinemia in the newborn, as well as some medicines, chemicals, and foods, can severely aggravate the clinical picture.

Pyruvate kinase deficiency is autosomal recessive, and the second most common red cell enzyme defect in neonates. The severity varies from hydrops fetalis to severe early-onset neonatal hyperbilirubinemia, and mild hyperbilirubinemia mimicking physiologic jaundice. For further details and information see Chapter 29.

### Red Cell Membrane Defects

The main clues that a neonate has a red cell membrane disorder are a family history, DAT-negative hemolysis (with a sensitive method), and an abnormal blood film. Red cell membrane electrophoresis from pretransfusion samples is diagnostic.

Hereditary spherocytosis is the most common red cell membrane defect. It occurs in 1 in 5000 live births in couples of North European ethnicity, but is less frequent in other population groups. It is inherited as an autosomal dominant trait.<sup>2</sup> Blood films have a similar appearance to those in ABO HDFN; the DAT is negative. Some neonates require 1 or 2 red cell transfusions during the first months of life.

Hereditary elliptocytosis is a more complex autosomal dominant disorder. In heterozygotes the only findings are elliptocytes in the blood smear. Neonates who have more than one mutation in a red cell membrane protein (homozygotes or compound heterozygotes) have severe, transfusion-dependent, hemolytic anemia. The blood film is characteristic, showing a high proportion of bizarre fragmented red cells and microcytes (mean corpuscular volume <60 fL). Red cell transfusion is often necessary until splenectomy can be carried out. For further details and information see Chapter 29.

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## Management of Immune-Mediated Hemolytic Disease of the Fetus and Newborn

### Surveillance and Tests

Antepartum surveillance and treatment are detailed in Chapter 26. After adequate intrauterine transfusion treatment, fetal erythropoiesis is suppressed, and the red cell mass maintained by compatible transfused cells. As a result, most of these neonates present neither neonatal jaundice nor anemia of clinical importance.

after birth, and very few require postpartum exchange transfusion. In contrast, late anemia is common in the newborn because of suppression of erythropoiesis caused by intrauterine transfusions and continued presence of hemolyzing maternal antibodies. Therefore, the neonates need follow-up with monitoring of hemoglobin, blood film, reticulocyte count, and bilirubin in order to determine when hemolysis has stopped and endogenous red cell production takes over.<sup>2</sup> In any consideration of red cell transfusions, the neonate's general condition, growth, and development are of major importance for defining the appropriate hemoglobin level for intervention (see Chapter 30). The Apgar score can be useful because it reflects important signs and symptoms associated with postpartum anemia (eg, color, tachypnea, tachycardia, lethargy).

In developing countries, antepartum treatment is mostly unavailable, and even antibody screening and ABO/Rh typing are often not performed in the pregnant mother. In such areas, the clinical picture can be more like that seen in developed countries in the 1960s, with serious anti-D HDFN (erythroblastosis, hyperbilirubinemia with grave anemia on the first postpartum day, and, in serious cases, hydrops fetalis or even stillbirth).

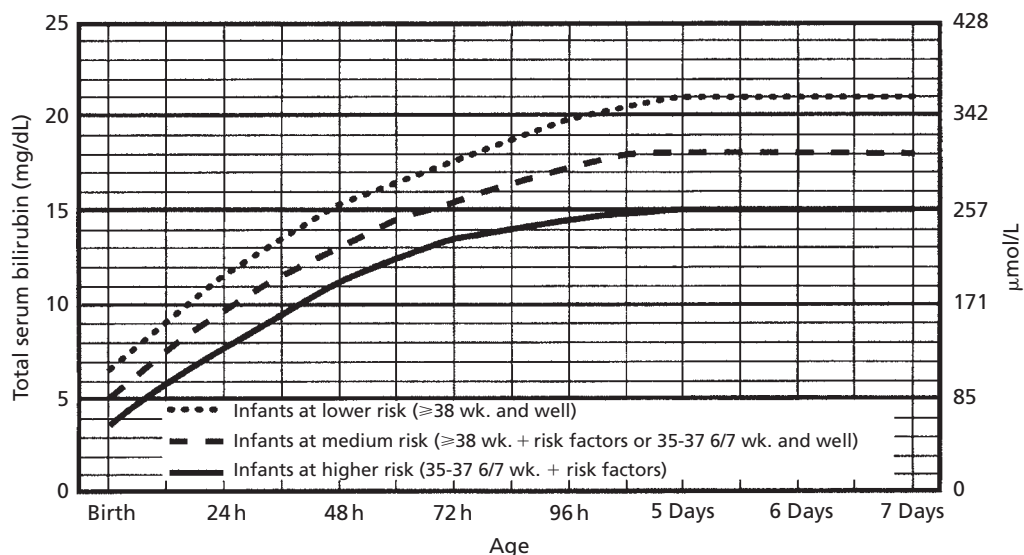
## Phototherapy

Improved delivery and understanding of phototherapy since its introduction in the late 1950s has had major effect on the clinical

treatment of HDFN. The efficacy of phototherapy is dependent upon the following factors<sup>16</sup>:

- Spectral qualities of the delivered light (wavelength range 400-520 nm, with peak emissions of 460 nm).
- Intensity of light (irradiance).
- Surface area receiving phototherapy.
- Skin pigmentation.
- Total serum bilirubin concentration at the start of phototherapy.
- Duration of exposure.

Modern phototherapy devices with high-intensity gallium nitride light-emitting diodes (LEDs) are small, efficient, easy to use, and ensure the correct distance between the neonate and the device.<sup>16</sup> The total radiation applied to a neonate has vastly increased and made "intensive phototherapy" an important alternative to exchange transfusion, even in the treatment of severe HDFN. "Intensive phototherapy" implies radiation in the blue-green spectrum (wavelengths  $\sim$  430-490 nm) of at least  $30 \mu\text{W}/\text{cm}^2/\text{nm}$  (measured at the infant's skin) and delivered to as much as possible of the infant's body surface area.<sup>17</sup> Figure 27-1 shows guidelines for "intense phototherapy" in neonates of 35 weeks or more with hyperbilirubinemia. The levels shown are approximations adapted to the infant's age and risk factors. Infants are designated as "higher risk" because of the potential negative effects of the conditions listed in the figure on albumin binding of bilirubin, the blood-brain barrier, and the susceptibility of brain cells to damage by bilirubin.<sup>17</sup> Low body



- **Use total bilirubin.** Do not subtract direct reacting or conjugated bilirubin.
- **Risk factors** are isoimmune hemolytic disease, G6PD deficiency, asphyxia, significant lethargy, temperature instability, sepsis, acidosis, or albumin  $<3.0 \text{ g/dL}$  (if measured).
- For well infants 35-37 weeks and 6/7 days, TSB levels can be adjusted for intervention around the medium risk line. It is an option to intervene at lower TSB levels for infants closer to 35 weeks and at higher TSB levels for those closer to 37 weeks and 6/7 days.
- It is an option to provide conventional phototherapy in hospital or at home at TSB levels 2 to  $3 \text{ mg/dL}$  ( $35\text{-}50 \mu\text{mol/L}$ ) below those shown, but home therapy should not be used in any infant with risk factors.

**Figure 27-1.** Guidelines for phototherapy in hospitalized infants of 35 weeks or more of gestation with hyperbilirubinemia, including that caused by HDFN. The guidelines refer to the use of "intensive phototherapy," which should be used if total serum bilirubin (TSB) exceeds the line indicated for each category. Used with permission from American Academy of Pediatrics.<sup>17</sup>

weight, particularly <1000 g, is an additional risk factor to be considered.

Today the “typical” neonate with HDFN presents in countries of high HDI as a well, term neonate with ABO incompatibility and a total serum bilirubin often in excess of 20 mg/dL but without significant anemia. “Intensive phototherapy” during the 4 to 6 hours it generally takes to organize an exchange transfusion most often results in a significant decrease in total serum bilirubin, thus frequently removing the indication for exchange transfusion.<sup>2</sup> When high-dose IVIG is given as adjuvant to phototherapy the indication for exchange transfusion is even further reduced.

## High-Dose IVIG

Since the beginning of the 1990s, several pilot studies have reported the effect of high-dose IVIG as adjuvant to standard therapy for HDFN. Two systematic reviews have performed a meta-analysis of the impact of high-dose IVIG on the use of exchange transfusion, comparing IVIG plus phototherapy with phototherapy alone.<sup>18,19</sup> The conclusion of both reviews was that IVIG significantly reduced the risk of exchange transfusion. However, the authors of the two systematic reviews differ with respect to their recommendation as to the use of high-dose IVIG. The Cochrane review<sup>18</sup> concludes that the number and quality of studies, and the number of infants included is too small to recommend the use of adjuvant IVIG in HDFN, and suggests further well-designed studies. However, the other review<sup>19</sup> supports the use of high-dose IVIG, and also shows that when IVIG is used in addition to phototherapy in HDFN, both duration of phototherapy and hospital stay are reduced to a point that equals the cost of the IVIG in the United Kingdom.

With the decline in the number of exchange transfusions, the opportunity for further large, well designed studies is limited. Although exchange transfusions are associated with a mortality of 2% and serious complications in 12% of the patients (Table 27-3), no serious side effects have been reported in neonates treated with phototherapy and high-dose IVIG. It must, however, be remembered that IVIG is a pooled plasma product, which before the introduction of adequate pathogen inactivation, was involved in several cases of viral transmission. Since 1994 there has been no transmission of blood-borne infections with pathogen-inactivated IVIG prepared from selected donors<sup>20</sup> (see also Chapter 19). Thus, further randomized studies as suggested by the authors of the Cochrane meta-analysis<sup>18</sup> may even be considered unethical.

Recently the IVIG Hematology and Neurology Expert Panels have recommended that high-dose IVIG be offered to neonates with HDFN as treatment for severe hyperbilirubinemia<sup>20</sup> and endorsed the recommendations outlined in the American Academy for Pediatrics guideline on the treatment of hyperbilirubinemia.<sup>17</sup> That guideline states:

“In isoimmune hemolytic disease, administration of IVIG (0.5–1.0 g/kg over 2 hours) is recommended if the total serum

bilirubin (TSB) is rising despite intensive phototherapy or the TSB level is within 2 to 3 mg/dL (34–51 μmol/L) of exchange level.

If necessary, this dose can be repeated in 12 hours.”

The exact action of IVIG in HDFN is still unclear, but following mechanisms are probably involved (see also Chapter 18):

- Prevention of phagocytosis of IgG-coated red cells by blocking of Fc receptors on cells in the reticuloendothelial system.
- Neutralization of maternal antibodies by anti-idiotypic antibodies in IVIG.
- Cytokine modifying actions of IVIG.

## Exchange Transfusions

Criteria used to assess the need for exchange transfusion include total bilirubin level, hemoglobin level, and clinical symptoms. Traditional guidelines suggest exchange transfusion in the following circumstances:

Within 12 hours of birth if

- Cord blood bilirubin concentration exceeds 3 to 5 mg/dL for preterm infants, 5 to 7 mg/dL for term infants, or the rate of increase is >0.5 mg/dL/hour.
- Severe anemia: hemoglobin <10 g/dl combined with hyperbilirubinemia.

After 24 hours of birth if

- Total bilirubin concentration >20 mg/dL or a bilirubin increase of >0.5 mg/dL/hour or hemoglobin <10 g/dL combined with hyperbilirubinemia.

Today, the effect of “intensive phototherapy” and reduction of hemolysis by high-dose IVIG treatment almost always results in a considerable decrease in bilirubin levels during the 4 to 6 hours

**Table 27-3.** Complications of Exchange Transfusion

### Vascular

- Embolization of air or thrombi
- Thrombosis

### Cardiac

- Arrhythmias
- Volume overload
- Cardiac arrest

### Electrolytes

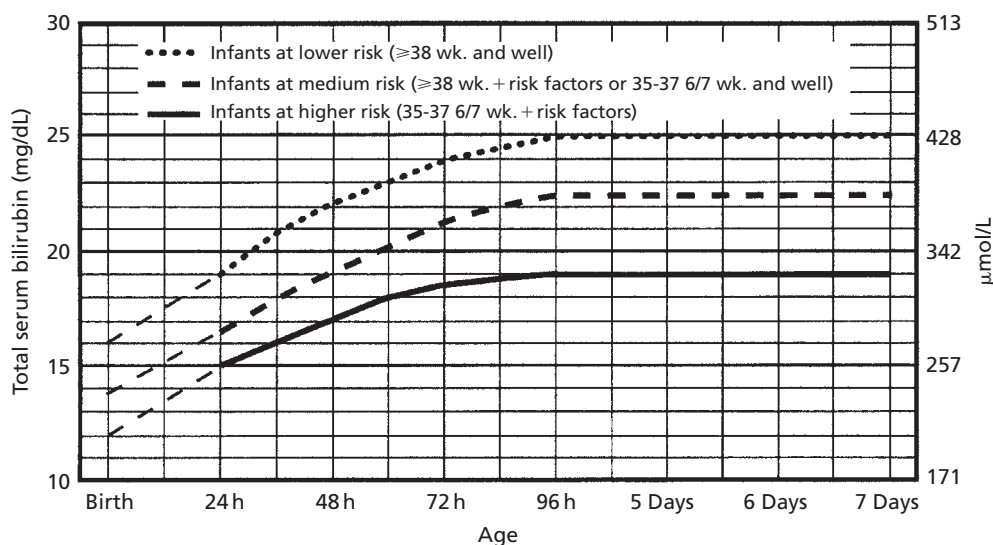
- Hyperkalemia
- Hyponatremia
- Hypocalcemia
- Acidosis

### Coagulation

- Overheparinization
- Thrombocytopenia

### Infectious

- Bacteremia
- Hepatitis
- Cytomegalovirus



- **The dashed lines for the first 24 hours** indicate uncertainty due to a wide range of clinical circumstances and a range of responses to phototherapy and high-dose IVIG.
- **Immediate exchange transfusion** is recommended if infant shows signs of **acute bilirubin encephalopathy** (hypertonia, arching, retrocollis, opisthotonos, fever, high pitched cry) or if TSB is  $\geq 5$  mg/dL ( $85 \mu\text{mol/L}$ ) above these lines.
- **Risk factors** are isoimmune hemolytic disease, G6PD deficiency, asphyxia, significant lethargy, temperature instability, sepsis, acidosis, or albumin  $< 3.0$  mg/dL (if measured).
- **Use total bilirubin.** Do not subtract direct reacting or conjugated bilirubin.
- If infant is well at 35-37 weeks and 6/7 days (median risk), TSB levels can be individualized for exchange based on actual gestation age.

**Figure 27-2.** Guidelines for exchange transfusion in infants 35 weeks or more of gestation with hyperbilirubinemia, including that caused by HDFN. The guidelines refer to the use of exchange transfusions, which should be used if total serum bilirubin (TSB) exceeds the line indicated for each category. During birth hospitalization, exchange transfusion is recommended if TSB rises to these levels

despite "intensive phototherapy." For readmitted infants, if TSB level is above the exchange level, repeat TSB measurements every 2 to 3 hours and consider exchange transfusion if the TSB remains above the levels indicated after "intensive phototherapy" for 6 hours. Used with permission from American Academy of Pediatrics.<sup>17</sup>

needed to set up an exchange transfusion. Thus, the clinical indication for exchange transfusion may be removed, but the need for simple transfusion increases.<sup>2</sup> Exchange transfusions are considered indicated when "intensive phototherapy," with or without IVIG, fails to adequately reduce bilirubin concentration or when the initial serum bilirubin places the infant at risk for kernicterus. Factors potentiating bilirubin toxicity include immune-mediated hemolysis, acid-base disturbances, asphyxia, free heme groups, and other byproducts of hemolysis or drugs that displace bilirubin from albumin and other plasma binding proteins.<sup>6</sup>

Figure 27-2 shows current guidelines for exchange transfusion in neonates of 35 weeks or more with hyperbilirubinemia.<sup>17</sup> The dashed lines for the first 24 hours on the figure indicate uncertainty due to the range of responses to phototherapy and to differences in clinical circumstances, thus emphasizing the importance of individualized treatment decisions. Treatment decisions after the first 24 hours are dependent upon gestational age, bilirubin concentration, the rate of its increase ( $> 0.5$  mg/mL/hour) and risk factors that have a potential negative effect on albumin binding of bilirubin, the blood brain barrier, and the susceptibility of brain cells to damage by bilirubin (see Fig 27-2). Immediate exchange transfusion is recommended if the

infant shows signs of acute bilirubin encephalopathy as detailed in Fig 27-2, or if total bilirubin is 5 mg/dL above the lines.

Exchange transfusions supply the neonate with compatible red cells and fresh plasma, while incompatible red cells, bilirubin, and maternal antibodies in plasma are removed. A standard exchange transfusion of twice the infant's blood volume reduces incompatible fetal red cells by about 85%; bilirubin and maternal antibody concentrations are reduced by 25% to 45%.<sup>6</sup> The amount of blood needed for an exchange transfusion is 170 mL/kg (200 mL/kg in preterm infants). An exchange transfusion consists of hemoglobin S-negative red cells that are compatible with maternal serum and/or plasma and are reconstituted with fresh frozen group AB plasma to obtain a hematocrit of 45% to 50%. The red cells should be leukocyte-reduced (to prevent transmission of cytomegalovirus), irradiated, and preferably  $< 5$  days old. Platelet addition is to be considered only if an infant's platelet count is seriously compromised or after multiple exchange transfusions.

In view of the declining number of exchange transfusions, and the risks involved with the procedure (Table 27-3) it is important that maternity and neonatal services develop, maintain, and teach appropriate written guidelines for the use of exchange transfusion in HDFN and the performance of the procedure itself.

## Conclusion

In immune-mediated HDFN, maternal IgG antibodies—usually related to the ABO, Rh, or Kell blood groups—cross the placental barrier, causing hemolysis of fetal red cells. HDFN caused by hereditary red cell disorders are less frequent. Although HDFN can be fatal, advances in diagnosis, treatment, and prevention have made immune-mediated HDFN increasingly controllable in developed countries. Diagnostic modalities include maternal assays, antepartum treatment, and routine prophylactic RhIG given to D-negative women at risk. Postpartum management includes treatment of hyperbilirubinemia and anemia. Hyperbilirubinemia is usually treated by means of “intensive phototherapy,” while adjuvant high-dose IVIG is given in order to reduce hemolysis. In addition, anemia is corrected by simple transfusion of red cells. These approaches have reduced the indications for exchange transfusion to very severe HDFN or when “intensive phototherapy” combined with IVIG fails to reduce hyperbilirubinemia to acceptable levels. Further improvement of phototherapy equipment, IVIG treatment, and possible administration of D-penicillamine and/or metalloporphyrins in order to reduce bilirubin formation<sup>21,22</sup> could reduce the need of exchange transfusions and change the spectrum of HDFN treatment even more.

While mortality and morbidity of HDFN have been greatly reduced in developed countries, HDFN still poses a serious challenge in many developing countries. In these countries with low HDI, introduction of maternal assays, routine prophylactic RhIG treatment, and affordable phototherapy should be encouraged. Because high-dose IVIG often will be beyond economic reach, phototherapy combined with drugs to reduce the postpartum formation of bilirubin could be of interest, and further studies with the administration of D-penicillamine<sup>21</sup> or metalloporphyrins<sup>22</sup> are to be encouraged.

## Disclaimer

The authors have disclosed no conflicts of interest.

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# Congenital Disorders of Clotting Proteins and Hypercoagulable States in Pediatrics

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Historically, inherited disorders of coagulation lead to severe, debilitating disability and early demise. Even 20 to 30 years ago, children afflicted with hemophilia and thrombosis suffered much with few safe and effective treatment options. Today, the treatment of pediatric coagulopathies consists of effective modalities that are primarily administered on an outpatient basis. There are life-saving treatment options exist even for some of the most difficult-to-treat hemophilia patients with inhibitor or children with inherited thrombophilia. Multicenter prospective clinical trials involving children with bleeding disorders, as well as those with thrombophilia, are being performed in order to make accurate recommendations of care. Although studies in children with thrombotic conditions are few, evolving treatments contribute to an exciting era of growth in pediatric coagulopathy.

## Hemophilia

The hemophilias form a group of inherited lifelong bleeding disorders caused by deficiency of Factor VIII (hemophilia A) or Factor IX (hemophilia B). Hemophilia A and hemophilia B have many features in common. Both are inherited as X-linked recessive traits and thus affect males almost exclusively. The incidence of hemophilia A is 1 in 5000 male live births, and that of hemophilia B is 1 in 30,000.<sup>1,2</sup> Hemophilia affects at least 20,000 persons in the United States alone. Results of a US epidemiologic survey indicated that 43% of hemophiliacs are classified as severe, with 26% classified as moderate, and 31% as mild.<sup>3</sup> There are, however, some reports of hemophilia A and B in females, which could be caused by inheritance of a gene for hemophilia from both parents or a new mutation. The genes for Factor VIII and Factor IX are on the long arm of the X chromosome in band Xq28 and Xq27, respectively.

Hemophilia A and B are clinically indistinguishable. Bleeding into joints and soft tissues is the hallmark of both conditions. Laboratory screening tests are the same in both disorders as well. Persons with hemophilia A or B characteristically have normal values for prothrombin time, platelet count, and platelet function analyzer testing (which has replaced the template bleeding time in many institutions), but have a greatly prolonged activated partial thromboplastin time (aPTT). The two disorders can be distinguished by performance of assays for Factor VIII and Factor IX coagulant activity. Hemophilia is worldwide in distribution and affects all racial groups.

## Clinical Features

In hemophilia A, Factor VIII coagulant activity is deficient or abnormal, whereas other components of the Factor VIII system—von Willebrand factor (vWF) and vWF antigen (vWF:Ag)—are normal. In general, clinical severity is correlated with the degree of Factor VIII deficiency. Persons with severe hemophilia have <1% (<0.01 IU/mL) Factor VIII activity. Severely affected persons have spontaneous bleeding into joints and soft tissues. Those with 1% to 5% of normal Factor VIII activity are classified as moderate, and have little, if any, spontaneous bleeding. Those with Factor VIII values of >5% to 40% (>0.05 to 0.40 IU/mL) have clinically mild disease, usually bleeding only with surgery or trauma.<sup>1</sup> The degree of Factor VIII deficiency is fairly consistent in individuals, and in affected members in the same kindred. “Normal” is 1 IU/mL of Factor VIII (100%), as defined by the current World Health Organization International Standard for Plasma Factor VIII [as distributed by the National Institute for Biological Standards and Control (NIBSC) in the United Kingdom].<sup>1</sup> Female carriers of the disease have variable factor levels; bleeding does not occur in those with near normal levels, but those with <50% levels may bleed more often than unaffected relatives or matched controls.<sup>4</sup>

Hemophilia B (Christmas disease) is characterized by subnormal Factor IX activity. Depending on the genetic defect, Factor IX deficiency may reflect a quantitative or qualitative abnormality in the Factor IX molecule. Several different subtypes of hemophilia B

have been described. In fact, genotype heterogeneity is quite marked; almost every family with hemophilia B has its own unique mutation.<sup>5</sup> Hemophilia B is far less common than hemophilia A, accounting for approximately 15% to 20% of cases of hemophilia. As in hemophilia A, clinical severity is correlated with the degree of Factor IX deficiency, with those with Factor IX levels of  $\leq 1\%$  generally having spontaneous bleeding into joints and soft tissues. The Leyden phenotype of hemophilia B is characterized by severe hemophilia in childhood that becomes mild after puberty.<sup>6,7</sup>

Joints are the classical bleeding sites in patients with severe hemophilia. Those with severe hemophilia have an annual average of 20 to 30 episodes of spontaneous or excessive bleeding after minor trauma. Some patients with severe disease have a milder clinical course. Coinheritance of the Factor V Leiden mutation (see below) occurs in a small proportion (4.4% in one series) of patients with severe hemophilia A.<sup>8</sup> The presence of this and other prothrombotic conditions partially counteracts the bleeding tendency of hemophilia, resulting in fewer bleeding episodes and a later onset of first bleeding.<sup>8-10</sup>

Clinical presentations may vary depending on the age of the patient. In the perinatal period, approximately 5% of such patients develop significant subgaleal or intracranial hemorrhage (ICH).<sup>11-13</sup> In a multicenter survey, symptomatic ICH occurred in 4% of 744 patients, with 40% occurring within the first week of life due to trauma as the major precipitating factor.<sup>11</sup> These hemorrhages may be complicated by seizures, psychomotor retardation and cerebral palsy.<sup>12</sup> In approximately 50% of undiagnosed hemophiliacs early bleeding occurs in association with circumcision.<sup>14</sup>

Children become symptomatic more often after the newborn period but within the first 2 years of life.<sup>15</sup> In one study, the first symptomatic bleeding leading to the diagnosis of severe hemophilia occurred at a median age of 0.9 year in children not carrying any prothrombotic risk factors, and 1.6 years in those carrying prothrombotic risk factors (eg, Factor V Leiden) ( $p = 0.01$ ).<sup>8-10</sup> The onset of disease occurs later in patients with moderate and mild hemophilia, sometimes as late as 22 months.<sup>16</sup> Mild hemophilia, in the absence of an informative family history, may go undetected for significant periods (age 14 to 62 years in one report).<sup>17,18</sup> With ambulation, bleeding episodes occur more often and in any location.

The factors that initiate hemorrhage are not known, and the onset of hemorrhage often is a random event, occurring either spontaneously or after minimal injury. Bleeding into a joint (hemarthrosis) is the commonest bleeding manifestation; the ankles are most commonly affected in children, and the knees, elbows, and ankles are more affected in adolescents and adults. Spontaneous hemarthroses are so characteristic of severe hemophilia as to be almost diagnostic.<sup>1,19</sup> Hemarthrosis is painful and physically debilitating, and is the initiating event in hemophilic arthropathy. One joint is usually affected at a time, often leading to a target joint, but multiple bleeding sites are common.<sup>20</sup> Repeated hemorrhages into joints initiate the

progressive destruction of a joint, leading to fibrosis of the joint with contractures, pain, and limitation of motion.<sup>21</sup> The number and type of hemarthroses correlate with function and future quality of life. Thus, orthopedic complications remain a serious problem.

Bleeding into muscles is the next most common manifestation, and most often affects the quadriceps, iliopsoas, and forearm muscles, and sometimes are large enough to compromise neurovascular structures and produce a compartment syndrome.<sup>22</sup> Other sites of bleeding include the nose (epistaxis), oral mucosa, gastrointestinal tract, and hematuria. Intracranial hemorrhage occurs in older children as well as neonates. In one study of children, the prevalence of ICH was 12%.<sup>23</sup>

### Genetics in Hemophilia

Hemophilia A and B can be “familial” or can occur as a “sporadic” mutation. Seventy percent of mild to moderate cases of hemophilia, 57% of severe hemophilia B cases, and 45% of severe hemophilia A cases are clearly familial on pedigree analysis. Regardless, in isolated cases of hemophilia the mother of the index case carries the mutation in 85% of the cases; however, the mother’s mother carried the mutation in only 11% of isolated cases. Isolated cases, referring to only one affected individual in a family, account for approximately 25% of severe hemophilia cases and 15% of mild hemophilia cases. These “isolated cases” do not include sporadic cases in which a sibling (second child) is also diagnosed with hemophilia even if there is no family history.<sup>24</sup> One study looked for the genetic origin of sporadic cases of hemophilia A. It found that the majority of these boys inherited their Factor VIII inversion mutations from a *de novo* mutation that originated in the male germ cells of the maternal grandfather.<sup>25</sup>

Neither Factor VIII nor Factor IX crosses the placenta; thus, the diagnosis of hemophilia A or B can be made at birth from a sample of cord blood drawn from a vessel on the fetal side of the placenta, after the infant is born. The blood should be collected in citrate (1:10 dilution) and sent immediately to the laboratory for a Factor VIII or IX assay. The diagnosis can also be made by cordocentesis at 18 to 20 weeks of gestation. Similarly, umbilical cord blood can be collected and tested for Factor VIII or IX activity. However, prenatal diagnosis of hemophilia is now accomplished almost exclusively through molecular biological techniques analyzing various polymorphic markers from DNA obtained from chorionic villus sampling. These modern techniques, including restriction fragment length polymorphism analysis, denaturing gradient gel electrophoresis, single-strand conformation polymorphism, DNA sequencing, and others, are accurate and can be obtained earlier in the pregnancy with less morbidity than cordocentesis.<sup>26</sup>

Although several mutations in the Factor VIII and Factor IX genes have been described,<sup>2,4-6</sup> an inversion in the intron 22 of the Factor VIII gene accounts for roughly 45% of cases of severe (not mild or moderate) hemophilia A.<sup>27</sup> Thus, the search for inversions of Factor VIII should be considered as the

first DNA diagnostic option in a person or a family with severe hemophilia A.<sup>28</sup> The inversion mutation can be detected by polymerase chain reaction amplification and other molecular techniques from a person with hemophilia A or from a possible carrier, or using fetal or chorionic villus cells. These techniques can be used to diagnose virtually 100% of the intron 22 inversions seen in severe hemophilia. In one study, however, genomic analysis techniques failed to detect the defect in approximately 4% of moderately affected hemophilia A persons and approximately 12% of mildly affected persons.<sup>29</sup>

Treatment of Hemostatic Abnormalities in Hemophilia

The optimal management of these patients is very complex, and requires the provision of preventive care, the use of replacement therapy, and treatment of complications of the disease and its therapy. During the first half of the 20th century, life for these patients was at best miserable because they all developed disabling arthropathy (usually before the age of 20), often leading to permanent disability. Hemophilia also had a high mortality rate from ICH. In the 1920s and 1930s, life expectancy in developed countries such as Finland and Sweden was only 8 to 11 years. As recently as 1960, life expectancy was <30 years.<sup>30-33</sup> Improvements in hemophilia care saw its share of setbacks, the most burdensome of which was the transmission of human immunodeficiency virus (HIV) and hepatitis C virus (HCV), both of which had a devastating effect on this community.<sup>34,35</sup> With replacement therapy came two further challenges—the development of inhibitors to Factor VIII or IX, and the rising costs of treatment. Because of the increasing costs of care, developing countries struggle to provide factor concentrates to

their patients. The challenge facing clinicians today is to maintain state-of-the-art care in an environment of rising treatment costs.<sup>36</sup>

Rapid advances within the last 30 years in the evolution of factor replacement products have been made (Table 28-1). The availability of factor concentrates, the emergence of comprehensive hemophilia treatment centers, and the widespread adoption of home-administered replacement therapy led to the early control of hemorrhages and reduced the musculoskeletal damage typical in patients with inadequately treated disease.<sup>19</sup>

Blood Component Therapy

Until the mid-1960s, fresh plasma or Fresh Frozen Plasma (FFP) were the only preparations that could be used to treat Factor VIII or Factor IX deficiency. Then, in 1965, Pool et al<sup>37</sup> described a simple method for concentrating Factor VIII in the form of a cold-insoluble precipitate (cryoprecipitate). By the late 1960s, the availability of Cryoprecipitated AHF had revolutionized the treatment of hemophilia A and made possible elective surgery and outpatient treatment of bleeding episodes.

Cryoprecipitates, prepared from single units of plasma, contain approximately 50% of the Factor VIII activity, vWF, and fibrinogen, as well as Factor XIII, from the starting unit of plasma. Thus, for purposes of calculation, a single bag of cryoprecipitate contains, on average, 100IU of Factor VIII and 0.2g of fibrinogen in a volume of 8 to 10 mL.

In the late 1970s and early 1980s, approximately half of the hemophiliacs in the United States or about 9300 people developed HIV from lyophilized concentrates and cryoprecipitates. In addition, about 80% of HIV-positive hemophiliacs were eventually found to have HCV.<sup>38</sup> As a result, cryoprecipitates

Table 28-1. Evolution of Hemophilia A and B Therapy

| Year | Hemophilia A                                                                     | Hemophilia B                                                                                |
|------|----------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|
| 1950 | Plasma infusion                                                                  | Plasma infusion                                                                             |
| 1966 | Cryoprecipitate infusion                                                         | —                                                                                           |
| 1975 | Lyophilized concentrates                                                         | Lyophilized prothrombin complex concentrates                                                |
| 1983 | Heat treatment of lyophilized products                                           | —                                                                                           |
| 1985 | —                                                                                | Virus attention by heat treatment of lyophilized concentrates                               |
| 1986 | Introduction of solvent/detergent treatment for virus inactivation               | Introduction of solvent/detergent treatment for virus inactivation                          |
| 1988 | Monoclonal-antibody-purified Factor VIII concentrates, eg, Monoclote             | —                                                                                           |
| 1990 | —                                                                                | Chromagrapic/monoclonal antibody purified plasma-derived Factor IX, eg, Mononine, Alphanine |
| 1992 | Recombinant first-generation Factor VIII concentrates, eg, Recombinate           | —                                                                                           |
| 1996 | —                                                                                | Recombinant Factor IX, eg, BeneFix                                                          |
| 2000 | Recombinant second-generation Factor VIII, eg, Kogenate FS, Helixate FS, Refacto | —                                                                                           |
| 2003 | Recombinant third-generation Factor VIII, eg, Advate                             | —                                                                                           |

were largely replaced by lyophilized Factor VIII concentrates and recombinant Factor VIII (rFVIII) concentrates. Regardless, cryoprecipitates are still used in many developing countries to treat persons with severe or moderately severe hemophilia A. Disadvantages of cryoprecipitates over commercially prepared concentrates include 1) a slightly increased risk of blood-borne viral disease transmission (particularly HCV), 2) a required storage temperature of  $<20$  to  $<30^{\circ}\text{C}$ , and 3) marked variation in the Factor VIII and vWF content of preparations.

By 1970, methods of fractionation had been developed to produce concentrates of both intermediate-purity Factor VIII and prothrombin complex concentrate (PCC), the latter of which contained the vitamin-K-dependent clotting factors (Factors II, VII, IX, and X) as well as proteins C and S. The availability of these lyophilized concentrates quickly led to home treatment (self-infusion) programs throughout the United States and abroad. For the first time, persons with hemophilia were not totally dependent on family members, hospital personnel, and emergency rooms. Bleeding episodes could be treated more promptly, less time was lost from work or school, and the incidence of progressive chronic musculoskeletal disease was decreased. However, it soon became apparent that many persons with hemophilia had developed hepatitis and had persisting alterations of liver function.<sup>39-41</sup> Some had progressed to cirrhosis. As many as 90% of hemophiliacs became seropositive to hepatitis B surface antigen.<sup>42</sup> Because of this serious complication, in 1980 a Factor VIII concentrate (Hemofil T, Hyland Laboratories, Glendale, CA) was developed that was heated in the dry state for 72 hours at  $60^{\circ}\text{C}$ . Studies in chimpanzees suggested that this method of heating destroyed hepatitis viruses; however, when Hemofil T was infused into previously untreated children with hemophilia, 84% developed non-A, non-B hepatitis (currently referred to as HCV).<sup>42</sup>

In the early 1980s, the first cases of acquired immunodeficiency syndrome (AIDS) in persons with hemophilia were noted. It quickly became apparent that this too was being transmitted by blood and blood components, including clotting factor concentrates.<sup>43</sup> Approximately 90% of persons who received Factor VIII concentrates and 55% of those who received PCCs prepared in the United States between 1979 and 1984 became seropositive for HIV. Fortunately, HIV proved to be heat labile.<sup>44</sup> Although the dry heating method used in production of Hemofil T did not prevent HCV, it did seem to destroy HIV. Thus, by late 1984 almost all hemophiliacs in the United States were receiving dry heat-treated concentrates. Additionally, by the spring of 1985, mandatory screening of all blood and plasmapheresis donors for HIV seropositivity was in place. These measures greatly improved the safety of plasma-derived Factor VIII and Factor IX concentrates in terms of HIV transmission. Since 1987, there has not been a new case of HIV or hepatitis in the North American hemophilia population.<sup>45</sup>

In addition to better virus-attenuated products, Factor VIII concentrates purified by using murine monoclonal antibodies and immunoaffinity chromatography became commercially available in 1987.<sup>46</sup> Several logs of virus were eliminated in the purification process. These monoclonal antibody-purified

products were further virus-attenuated by pasteurization or by solvent/detergent treatment.

Creutzfeldt-Jakob disease (CJD) was another potential threat for the hemophilia community. Although there have been no documented instances of transmission of CJD or variant CJD, the mere possibility of such transmission has been cause for further concern among hemophilic patients and their physicians.<sup>38</sup> Thus, the use of safer alternatives to blood concentrates/derivatives was explored.

### Recombinant Factor VIII

Despite improved methods of donor screening and virus attenuation of plasma-derived clotting factor concentrates, the transmission of certain blood-borne viruses remained a concern. Thus, recombinant clotting factor concentrates have had great appeal. After scientists described the molecular cloning of a complementary DNA (cDNA)-encoding human Factor VIII<sup>47</sup> and the expression of human Factor VIII from recombinant DNA clones,<sup>48</sup> scale-up, purification, and standardization were rapidly accomplished. Preliminary clinical trials in humans began in 1987.

In patients with hemophilia A, the original, “first-generation” full-length rFVIII products have demonstrated the same pharmacokinetics and clinical effectiveness as plasma-derived Factor VIII products.<sup>49,50</sup> Despite the cost of rFVIII, many patients (especially those who are HIV seronegative) now have been switched from plasma-derived to rFVIII, because of the added margin of safety. Still, there were concerns among some that human serum albumin was in these rFVIII products (and was, in fact, the major component in the final products) and *might* transmit a blood-borne disease.

The “second generation” of rFVIII products that were developed included truncated molecules. Because the heavily glycosylated B domain seemed to be dispensable for the hemostatic activity of Factor VIII,<sup>51</sup> a B-domain-deleted (BDD) form of Factor VIII was developed.<sup>31,32</sup> This much smaller molecule is secreted more efficiently by Chinese hamster ovary (CHO) cells, is less prone to proteolytic degradation, and no serum albumin is needed for stabilization of the final product. B-domain-deleted rFVIII has proven to be effective in a wide variety of situations (acute bleeding episodes, prophylaxis, home treatment, surgery, etc), is safe, and is well tolerated.<sup>52</sup>

The only “problem” with BDD rFVIII is that usual, one-stage (aPTT-based) Factor VIII assays give roughly 50% of expected values in the recipient’s plasma.<sup>52-54</sup> There are marked differences in the phospholipid requirement in the assay of BDD rFVIII compared to the other Factor VIII preparations. Thus, if one is monitoring Factor VIII levels in a patient with hemophilia A who is receiving BDD rFVIII, one-stage Factor VIII assays will appear to be lower than expected (calculated). On the other hand, chromogenic substrate assays, although infrequently performed by US clinical laboratories, will give expected recovery values. Also, if one uses a small test vial of BDD rFVIII as the Factor VIII standard in the one-stage assay, results will be as expected. Small

test vials of BDD rFVIII (prepared by the (NIBSC) are available for this purpose.

Another “second-generation” rFVIII product is a full-length preparation formulated with sucrose, rather than human serum albumin.<sup>55</sup> The purification process for this product (Kogenate FS, Bayer Healthcare, West Haven, CT) eliminates the need for the addition of albumin. A solvent/detergent treatment step has also been added during purification. In 2003, a third-generation Factor VIII product (Advate, Baxter, Deerfield, IL) was released. This product has no human or animal proteins either in the initial stages of preparation or in the final formulation.

Dosage and Administration of Factor VIII

Factor VIII concentrates are routinely used for treating acute bleeding episodes in persons with moderate or severe hemophilia A. Factor replacements may be given either “on demand” (ie, after a bleeding episode to treat the hemorrhage) or as “prophylaxis” (ie, scheduled infusions to prevent or decrease the frequency and intensity of hemorrhages). The most common indications for treatment are acute hemarthrosis and intramuscular bleeding. Such events should be treated promptly to prevent or reduce complications (such as chronic joint disease) and to minimize the need for additional infusion of clotting factor (Table 28-2). In addition, Factor VIII concentrates are often used for prophylaxis (to prevent bleeding), for surgical coverage, and for immune tolerance induction (ITI) in persons with Factor VIII inhibitors.

In calculating the dose, it can be assumed that 1 IU of Factor VIII/kg of body weight will raise the patient’s Factor VIII level by 2% (0.02 IU/mL). Thus, if the patient’s baseline Factor VIII level is 0.01 IU/mL, a dosage of 20 IU/kg would be expected to

raise the level to 40% (0.40 IU/mL). The half-life of Factor VIII is 10 to 12 hours but may be shorter if the recipient is febrile, is bleeding extensively, has a Factor VIII inhibitor, or is a small child.

Serious, life-threatening bleeding into the central nervous system or intraoperative and postoperative bleeding may be treated with a continuous infusion of Factor VIII. After an initial bolus of 40 to 50 IU/kg (which will raise the patient’s Factor VIII level to 80% to 100%), a continuous infusion could be started. An initial rate of 3 to 4 IU/kg/hour should be subsequently adjusted as indicated by the recipient’s Factor VIII level. In general, a rate of 2 IU/kg/hour will achieve a Factor VIII level of 25%; 3 IU/kg/hour will yield a 50% level; and 4 IU/kg/hour will result in a 75% level. However, these levels should not be assumed, and daily monitoring should be performed. If continuous infusion becomes a logistical issue, then twice a day bolus infusions may be substituted to keep a trough Factor VIII level >50%.

Prophylaxis

Chronic hemophilic arthropathy, resulting from repeated bleeding into joints, is the major cause of morbidity in persons with severe hemophilia. In those whose Factor VIII or IX levels are less than 0.01 IU/mL, bleeding episodes occur approximately 20 to 30 times per year.<sup>1</sup> Primary prophylaxis, which is aimed at preventing spontaneous hemarthroses, was begun in Malmö, Sweden, over 35 years ago.<sup>57</sup> All boys with severe hemophilia currently seen at the Malmö center start prophylaxis when they are 1 to 2 years old. Most standard prophylaxis regimens aim to keep the factor level >1%.<sup>58</sup> For Factor VIII deficiency, the standard implies infusions three times per week; for Factor IX replacement, the standard implies two infusions per week because of

Table 28-2. Management of Bleeding Episodes in Hemophilia: Suggested Recommendations

| Type of Bleeding | Examples                                                                                               | Factor VIII/IX Levels Desired* | Duration of Therapy                                     |
|------------------|--------------------------------------------------------------------------------------------------------|--------------------------------|---------------------------------------------------------|
| Minor            | Uncomplicated hemarthroses, superficial muscle or soft tissue bleeding                                 | 20-40%                         | 1-3 days                                                |
| Moderate         | Intramuscular or soft tissue bleeding with dissection; mucous membranes; dental extractions; hematuria | 30-60%                         | 2-7 days                                                |
| Major            | Pharynx; retropharynx; retroperitoneum; central nervous system (CNS) surgery                           | 60-100%                        | 5-14 days; CNS bleeding—6 weeks to months to indefinite |
|                  | Postoperative management                                                                               | 60-80%                         | 7-14 days                                               |
|                  | Prophylaxis therapy— administered 3 or 4 times/week†                                                   | Trough factor >1%              | ??                                                      |

\*The required dosage for Factor VIII is determined using the formula:  
Required units = Body weight (kg) × Desired Factor VIII Rise (%) × 0.5 IU/kg.  
The required dosage for Factor IX is determined using the formula:  
Required units = Body weight (kg) × Desired Factor VIII Rise (%)  
†One study<sup>56</sup> showed that once-a-week prophylaxis may be feasible in some patients.

the longer half-life of Factor IX.<sup>59</sup> Although venous access may be a problem in younger children, central venous catheters are often used. With such devices the risk of bleeding or infection is generally low but must be considered. Another historical concern has been the propensity for increased Factor VIII inhibitor formation with the increased number of infusions given in prophylaxis. However, recent reports suggest that there may be a benefit resulting in decreased inhibitor formation with prophylaxis.<sup>58</sup>

Prophylactic treatment with recombinant Factor VIII has been shown to prevent joint damage in young boys with severe hemophilia A. A prospective randomized, multicenter study conducted in the United States enrolled 65 boys <30 months of age with severe hemophilia A between August 1996 and March 2000. The boys were randomly assigned to receive either prophylactic Factor VIII dosing at 25 IU/kg every other day or episodic therapy only at the time of a joint hemorrhage with an initial dose of 40 IU/kg and 20 IU/kg 24 and 72 hours after the first dose. Parents were also encouraged to re-treat up to 4 weeks with 20 IU/kg every other day until the symptoms of joint hemorrhage resolved. Radiography, magnetic resonance imaging (MRI), and a physical examination scoring system were used to assess joint damage. The prophylaxis group had a significant decrease in structural joint damage as assessed by MRI. An 86% relative risk reduction in joint damage as assessed by the scoring systems was noted as compared to on-demand therapy.<sup>60</sup>

### Coagulation Concentrates Available for the Treatment of Hemophilia B

Until late 1990, intermediate-purity PCCs were the mainstay of treatment for persons with hemophilia B. They contain the vitamin-K-dependent clotting Factors II, VII, IX, and X, as well as proteins C<sup>61</sup> and S. In addition to nonactivated factors, PCCs contain some coagulation factors in activated forms as well. This occasionally results in disseminated intravascular coagulation (DIC) or thromboembolic complications in recipients of these products. The risk of DIC and thromboembolism is greatest in recipients who have sustained crush injuries or extensive soft-tissue bleeding, or who have undergone orthopedic surgery and are immobile. In such situations, thromboplastic materials are released into the circulation. The risk is also enhanced in persons with hepatocellular disease, because clotting intermediates are not optimally cleared from the circulation, and antithrombin levels are often low.<sup>62</sup> Because of the potential, albeit rare, thrombotic complications of activated PCCs (aPCCs), most physicians avoid concurrent treatment with aPCCs and antifibrinolytics.<sup>63</sup> Recent studies using PCCs as prophylaxis in the treatment of hemophiliacs with inhibitor (see below) demonstrated no prothrombotic complications at doses of 75 to 100 U/kg every other day to three times per week.<sup>64</sup> The only aPCC (FEIBA-VH, Baxter Healthcare) currently available and licensed for use in the United States is now relegated to treatment as a bypassing agent for patients with inhibitors.<sup>64</sup>

In view of the thrombogenic potential of PCCs, there was an obvious need for high-purity Factor IX concentrates. These

so-called coagulation Factor IX concentrates are considerably more expensive than PCCs; however, they are far less thrombogenic.<sup>65</sup> These high-purity virus-attenuated Factor IX concentrates and recombinant concentrates are recommended for use in neonates and children, in persons with hemophilia B undergoing surgery (particularly orthopedic surgery), in those with crush injuries or large intramuscular hemorrhages, in those with hepatocellular dysfunction, and in anyone with a history of thrombotic problems after receiving PCCs (ie, in any high-risk situation for thrombosis or DIC)—in fact, they are the preferred products for anyone with hemophilia B.

### Recombinant Factor IX

The CHO cell line used in the manufacture of recombinant Factor IX is co-transfected with a human Factor IX cDNA expression plasmid and a cDNA expression plasmid that encodes an engineered form of the paired amino-acid-cleaving enzyme, which improves the processing efficiency of profactor IX expressed in CHO cells.<sup>66,67</sup> No albumin is used, and no human plasma, animal plasma, or animal-derived protein is used in its manufacture or purification.<sup>68</sup> A reformulation (BeneFix, Wyeth, Collegeville, PA) now exists that seems to exhibit equal efficacy to the original preparation with a more concentrated formulation. There were no instances of inhibitor development, allergic reactions, or thrombosis in 34 patients tested.<sup>69</sup>

### Dosage and Administration of Factor IX

Because Factor IX is a smaller molecule than Factor VIII and diffuses from intravascular to extravascular sites, a larger dose must be given to achieve the same concentration in the circulation. Whereas a dose of Factor VIII of 1 IU/kg will raise the serum Factor VIII level by 0.02 IU/mL (2%), the same dose of (plasma-derived) Factor IX will raise the circulating Factor IX level by only 0.01 IU/mL (1%). As in the case of Factor VIII deficiency, in hemophilia B the recommended dosage for treatment of bleeding depends on the nature and severity of the bleeding episode. For most situations, dosages should be calculated to raise the Factor IX level to 0.2 to 0.3 IU/mL (20%-30%). However, larger doses are recommended for treatment of serious, life-threatening bleeding episodes and for surgery.

Recombinant Factor IX does have a decrease in recovery of the product by ~50% to 72% with respect to plasma-derived Factor IX.<sup>70</sup> However, the recombinant preparation has also been shown to exhibit a longer half-life of 13.7 vs 12.9 hours for the plasma-derived product to the extent that in three of the 13 patients in this recovery study, less recombinant product was infused to maintain the same baseline level of 2%.<sup>71</sup> The difference in recovery results from a simple difference in the posttranslational modification, or namely, the differences in sulfation of tyrosine 155 and phosphorylation of serine 158, residues that play a role in the clearance of Factor IX.<sup>70</sup> Thus, it is recommended that one use a somewhat larger dose of BeneFix than one would of a plasma-derived Factor IX product. The product package insert recommends the following calculation of dosage: number of

Factor IX units required = body weight (kg)  $\times$  desired Factor IX increase (%)  $\times$  1.2. However, one should be aware that there are wide variations in recovery among individuals. Infants and children tend to have lower recovery rates than adults.<sup>68</sup>

### **Surgery in Persons with Hemophilia B**

Through the 1980s many hemophilia centers avoided elective surgery (particularly orthopedic surgery) in persons with hemophilia B because of the risk of DIC and thromboembolic complications. As noted earlier, the risk is greatest in persons undergoing extensive orthopedic surgery on the lower extremities, because there is release of thromboplastic materials into the circulation and the patient is likely to be immobile. Now that less thrombogenic plasma-derived Factor IX preparations [Mononine (CSL Behring, King of Prussia, PA) and Alphanine SD (Grifols, Los Angeles, CA)] and recombinant Factor IX (BeneFix) are available, these should be used during and after surgery. Continuous infusion of BeneFix can be used safely and efficaciously to control bleeding in the perioperative period with little to no thrombotic side effects. The stability is maximized (90%) up to 14 days when stored at 4°C with the addition of 4  $\mu$ /mL of unfractionated heparin (UFH). The addition of the UFH also prevents thrombophlebitis, a known complication of continuous infusion.<sup>72,73</sup>

### **Other Ancillary Therapeutic Options**

#### ***Desmopressin***

Desmopressin (DDAVP) is the treatment of choice for persons with mild hemophilia A<sup>74</sup> whenever an approximately three-fold increase in Factor VIII is sufficient to control bleeding. This synthetic agent effects a three- to fivefold (average of threefold) increase in Factor VIII and vWF:Ag 30 to 60 minutes after administration.<sup>75</sup> It shortens the bleeding time and the aPTT. However, only a fraction of mild hemophiliacs and patients with von Willebrand disease (vWD) respond to this agent so a trial of desmopressin is important to confirm a response before a hemostatic challenge. The recommended dosage is 0.3  $\mu$ g/kg given intravenously.<sup>76</sup> The levels of Factor VIII (and vWF) generally remain elevated for 8 to 12 hours and the dose can be repeated at 8- to 12-hour intervals. If repeated doses are given in rapid succession, many (but not all) persons develop tachyphylaxis (a diminishing response). This is thought to reflect a depletion of the storage sites for Factor VIII and vWF. Desmopressin is remarkably free of undesirable side effects. In most persons, side effects are limited to facial flushing and a feeling of facial warmth. The antidiuretic properties of the drug seldom cause a problem. However, if repetitive doses of desmopressin are used along with large amounts of intravenous fluids, fluid and electrolyte balance must be carefully monitored to avoid hyponatremia and water intoxication. This precaution is particularly important in infants, small children, and elderly patients. In fact, when desmopressin is given to small children, fluid intake during the next 18 hours should be limited to avoid the possibility of water intoxication. Several formulations of desmopressin are available. The highly

concentrated intranasal spray is ideally suited for outpatient and home use, but patients must be counseled not to use the spray more than twice daily and not more than for 3 to 4 consecutive days.<sup>77</sup> For patients weighing <50 kg, one spray (in one nostril) is recommended, while for those weighing >50 kg, two sprays (one in each nostril) should be given. The prescription must be specific for the metered dose pump that delivers 0.1 mL (150  $\mu$ g of desmopressin) per actuation.

#### ***Antifibrinolytic Agents***

$\epsilon$ -Aminocaproic acid and tranexamic acid are antifibrinolytic agents that inhibit plasminogen activation. These agents are useful adjuncts in certain situations, to prevent lysis of a clot that has already formed as a result of specific replacement therapy. The oral mucosa has significant fibrinolytic activity, and antifibrinolytic agents are particularly useful in the control of bleeding in the oral cavity (eg, extraction of permanent teeth, lacerations of the tongue and mouth, and oral surgery).<sup>78</sup> The recommended dosage of  $\epsilon$ -aminocaproic acid is 75 mg/kg every 4 to 6 hours orally, after a loading dose of 100 mg/kg; the dose of tranexamic acid is 25 mg/kg every 6 to 8 hours. For invasive dentistry, antifibrinolytic therapy should be started the evening before the procedure and be continued for 7 to 10 days.

Swedish investigators<sup>79</sup> have demonstrated that transfusion requirements and postoperative bleeding after oral surgery in patients with hemophilia can be significantly reduced by the use of a tranexamic acid mouthwash in addition to systemic antifibrinolytic treatment. The mouthwash is prepared from 10% tranexamic acid for injection, diluted with sterile water. Sindet-Pederson et al<sup>79</sup> recommend the use of 10 mL of 4.8% tranexamic acid solution for 2 minutes four times daily.

#### ***Hepatitis B Vaccine***

In view of the risk, albeit very slight, of posttransfusion hepatitis, all persons with hemophilia (and without, according to the American Academy of Pediatrics) should be immunized against hepatitis B. Although all units of blood or plasma collected are tested for hepatitis B surface antigen, such testing may not be 100% effective in screening out all units that might transmit hepatitis B, and the risk is multiplied substantially in the case of plasma-derived clotting factor concentrates in which plasma from 2500 to 20,000 plasma donors is used to produce a single lot of concentrate. Vaccination against hepatitis B (with recombinant hepatitis B vaccine) should be given subcutaneously as soon as the diagnosis of hemophilia has been made.

#### ***Hepatitis A Vaccine***

Hepatitis A vaccine is also available, and is recommended for children (or any seronegative persons) with hemophilia 2 years of age or older.<sup>74</sup> It can be given subcutaneously,<sup>80</sup> which is preferred over intramuscular injections because of the tendency to develop muscle bleeding with intramuscular vaccination.<sup>81</sup> The Centers for Disease Control and Prevention did investigate the seroconversion to hepatitis A or B of people with blood disorders

and found no seroconversions that could be attributed to blood components.<sup>82</sup>

### **Avoidance of Drugs that Can Cause Platelet Dysfunction**

Certain drugs, aspirin in particular, induce platelet dysfunction that can aggravate the bleeding tendency in persons with hemophilia (or another underlying abnormality of hemostasis). Joint or soft-tissue bleeding is often painful. If aspirin or an aspirin-containing compound is taken to relieve that pain, the bleeding tendency may worsen, and a vicious cycle may ensue. This effect of aspirin is caused by an irreversible inhibition of platelet cyclooxygenase, which inhibits prostaglandin synthesis. Thus, aspirin and all aspirin-containing compounds should be avoided by people who have underlying coagulation disorders. Acetaminophen is a good alternative to relieve mild pain in addition to rest, cooling, and elevation. Other drugs that induce platelet dysfunction include antihistamines, phenothiazines, and nonsteroidal antiinflammatory agents such as indomethacin and ibuprofen. Recently, celecoxib and other cyclooxygenase 2 (COX-2) inhibitors known to exhibit antiinflammatory and antiangiogenic properties have been effectively utilized for analgesia in hemophilic arthropathies. COX-2 inhibitors do not interfere with platelet function as do the more traditional nonsteroidal antiinflammatory drugs.<sup>83</sup>

### **Treatment of Hemophilia with Inhibitors**

Approximately 20% to 35% of persons with hemophilia A develop inhibitor antibodies to Factor VIII, measured in Bethesda units (BU). A lesser percentage (between 2% and 5%) of persons with hemophilia B develop Factor IX inhibitors. The development of inhibitors (antibodies, primarily IgG) currently represents one of the most serious complications of hemophilia.<sup>84</sup> These antibodies complicate bleeding episodes because of diminished responsiveness to factor concentrates and increase the risk of uncontrollable bleeding, disability, and premature death. In addition, life-threatening symptoms of anaphylaxis can occur in about 5% of patients with Factor IX inhibitors and in some cases, development of a nephrotic syndrome has been reported.<sup>85</sup> The latter is seen, especially, in those individuals whose severe hemophilia B results from a large deletion, frameshift mutation, or stop codon in the Factor IX gene and occurs whenever a Factor IX-containing product is infused.<sup>86-88</sup> Inhibitors are much less common in patients with mild or moderate disease. Inhibitors typically develop in early childhood, within the first 10 to 20 exposure days to exogenous factor concentrates. Compared with hemophilia patients without inhibitors, patients with inhibitors experience bleeding that is harder to control once it starts. Not surprisingly, progressive joint disease and significant mobility impairments are far more prevalent in patients with inhibitors than in those without. Soucie et al<sup>89</sup> analyzed joint range of motion (ROM) data on 2378 children (ages 2 to 19 years) with severe hemophilia, 186 of whom had an inhibitor titer of  $>0.5$  BU at the time the joint measurements were made. Those with a measurable inhibitor had a twofold greater loss of joint ROM

(expressed as a percentage of normal ROM) when compared with those with inhibitor levels  $<0.5$  BU. In an Italian study of older inhibitor patients (mean age 36 years, range 15 to 65), it was noted on study entry that 80% were physically disabled,  $>60\%$  had measurable flexion contractures, and over 70% had mobility impairments.<sup>90</sup> These studies suggest that predictable and progressive impairment in joint function in inhibitor patients begins in childhood and worsens with the passing of time, with older adults experiencing significant orthopedic disabilities.

Many persons with hemophilia and inhibitors (approximately 50%) have a high response titer with  $>5$  BU/mL upon exposure to Factor VIII. These high-responder patients are particularly difficult to treat.<sup>91</sup> The remainder of hemophiliacs with inhibitors have a low-response inhibitor with  $<5$  BU/mL.<sup>86</sup> Approximately one-third of inhibitors (usually low-titer inhibitors) are transient.<sup>87,88</sup>

### **Inhibitors and Type of Replacement Product Used**

The early studies of the first-generation recombinant Factor VIII products demonstrated inhibitor antibodies in previously untreated patients (PUPs). This raised some concern, but as the trials progressed it became apparent that neither product was any more antigenic than plasma-derived Factor VIII.<sup>92,93</sup> A more recent PUP trial<sup>94</sup> studied various plasma-derived vs first- and second-generation recombinant products over a 23-year period with 72 hemophilia A patients enrolled. Thirty-one percent of the patients developed inhibitors (43% of the severely affected patients and only 8% of the moderately affected). Fifty-one patients were treated with plasma-derived products and 21 were treated with recombinant products. There was no significant difference in high-titer ( $>5$  BU) inhibitor development between the two groups.<sup>94</sup> In another PUP study<sup>95</sup> with BDD rFVIII, 33 of 101 patients (all severely affected) developed inhibitors to Factor VIII after a median of 12 exposure days. Seventeen of the 33 patients who developed an inhibitor had peak values of  $<5$  BU, while 16 had peak values of  $\geq 5$  BU. Eight of the 33 inhibitors were transient, disappearing despite “on demand” treatment with BDD rFVIII. Twelve additional patients (most high-responder inhibitor patients) had a good response to immune tolerance induction (ITI) with BDD rFVIII, with their latest inhibitor titers being negative.<sup>52</sup> These results are very similar to those seen with the two full-length rFVIII preparations.<sup>95</sup> Inhibitor development follows a typical pattern; the highest risk is within the first 20 days of exposure to Factor VIII.

The management of patients with Factor VIII inhibitors has two important aspects. The first is treatment for bleeding, and the second involves attempts to eliminate the inhibitor. The management of patients with Factor IX inhibitors involves a third important consideration: the monitoring for and management of allergic reactions or frank anaphylaxis to Factor IX concentrates and Factor IX-containing agents.<sup>96</sup>

Bleeding in patients with low inhibitor titers ( $\leq 5$  BU) often can be treated with Factor VIII concentrates in usual or somewhat increased dosages, because anamnestic responses are



minimal and hemostatic levels of Factor VIII can be achieved. However, other approaches must be used in high responders. If the inhibitor titer is >5 BU, infusion of large doses of human Factor VIII concentrates often results in a marked increase in the inhibitor titer (anamnestic response) without controlling the bleed.<sup>63</sup> The choice of treatment depends on several variables: the patient's inhibitor concentration, whether he is naive to plasma-derived products, the degree of cross-reactivity of the inhibitor to porcine Factor VIII, the nature and extent of bleeding, product availability, and the experience and preference of the medical personnel involved.<sup>86</sup>

### Prothrombin Complex Concentrates

Prothrombin complex concentrates and aPCCs have been used extensively for the treatment of bleeding in patients with inhibitors since 1972 and until recently have been the mainstay of treatment for joint and soft-tissue bleeding. They can be used in both Factor VIII and Factor IX inhibitor patients. The so-called nonactivated or standard PCCs have been largely replaced by the purposely activated products, particularly FEIBA-VH (Baxter). Although they are not always effective, there is no readily available laboratory test for monitoring the recipient, and no one has demonstrated convincingly how they work,<sup>97,98</sup> many clinicians use aPCCs.<sup>86</sup> The recommended dose of FEIBA is 50 to 75 IU/kg every 12 to 24 hours.<sup>64</sup> If necessary, this dosage can be repeated for a total of 3 to 5 days. However, the use (particularly for traumatic injuries) of frequent repetitive doses should be avoided and total doses should not exceed of 200 U/kg/day, because of the thromboembolic complications referred to earlier. Multiple studies have demonstrated the efficacy of FEIBA in the control of bleeding in patients with inhibitors. FEIBA has been used in treating acute bleeding, in perioperative control of bleeding, and most recently in prophylaxis for patients with inhibitors.<sup>59,64,99</sup>

### Porcine Factor VIII Concentrates

Factor VIII inhibitors exhibit varying degrees of species specificity. Most human Factor VIII inhibitors destroy human Factor VIII to a greater degree than Factor VIII from another species. Polyelectrolyte porcine Factor VIII was a highly purified freeze-dried porcine Factor VIII concentrate that had a much lower incidence of side effects than the older porcine preparations that were largely abandoned in the 1960s. Polyelectrolyte porcine Factor VIII was licensed in the United States in 1986 for life- and limb-threatening emergencies. Porcine Factor VIII may be ineffective for hemophilia patients with inhibitors because of human antibody cross-reactivity or development of porcine Factor VIII antibodies. Allergic reactions are generally mild but can be as serious as anaphylaxis. Either allergic or pyrogenic reactions are seen in as many as 40% of cases. In addition, viral contamination, particularly with parvovirus B19, is limiting the availability of porcine Factor VIII.<sup>63</sup> On the other hand, polyelectrolyte porcine Factor VIII, unlike PCCs and aPCCs, permits the measurement of Factor VIII levels in the recipient, and it has proved lifesaving in many serious situations. It is no longer produced because of

parvovirus infection of the swine colony that was used to manufacture this product. A recombinant version is currently undergoing clinical trials.

### Recombinant Factor VIIa

The development of recombinant Factor VIIa (rFVIIa) has been an important landmark in the management of hemophilia patients with inhibitors. The drug (Novo Seven, Novo Nordisk, Copenhagen, Denmark) has been used with clinical success in hemophilic patients with inhibitors who had life-threatening bleeding episodes,<sup>98</sup> in patients with acute bleeding that was not life- or limb-threatening, as prophylaxis for joint bleeding, and as prophylaxis in elective surgical procedures.<sup>100-102</sup> Monroe et al<sup>103</sup> demonstrated that concentrations of Factor VIIa much higher than those found normally in the circulating blood are able to mediate a tissue-factor-independent conversion of Factor X to Factor Xa on a phospholipid surface. rFVIIa induces hemostasis in the absence of Factor VIII and Factor IX, probably by enhancing thrombin generation on the thrombin-activated platelet surface. The rFVIIa binds to the platelet membrane. Factor VIIa, in concentrations of 50 to 100 nmol/L, then proceeds to activate Factor X on the platelet surface in the absence of Factors IX and VIII. This thrombin activation recruits more platelets and enhances the platelet-to-platelet adhesion. In addition, the thrombin activation leads to a tighter fibrin clot structure by recruiting Factor XIII.<sup>104</sup>

Since the first human patient's successful treatment with rFVIIa, during and following open synovectomy,<sup>105,106</sup> many clinical trials with rFVIIa have been conducted. These included pharmacokinetic studies (which documented the short half-life of the product, 2 to 3 hours),<sup>107</sup> dose-finding studies, home treatment studies,<sup>108</sup> and multiple surgical studies.<sup>100,109</sup> In most of these studies, as well as in a large compassionate-use database, rFVIIa has been given by intravenous (IV) bolus injections. The recommended dose is 90 µg/kg of body weight per dose, with repeat dosing (if necessary) given every 2 to 3 hours for the first 24 hours (or more), with increasing intervals of 3 to 6 hours thereafter. However, the clearance rate varies among individuals, with some children less than 15 years of age having three times the clearance rate achieved in adults.<sup>110</sup>

Adverse effects found with the use of rFVIIa are rare with the exception of lack of efficacy. The Hemophilia and Thrombosis Research Society (HTRS) Registry reported on the use of rFVIIa in treating 719 bleeding episodes in 50 hemophilia patients. There were nine reported adverse events in five of the patients. Eight of the nine events were "decreased therapeutic response." The ninth reported adverse event was a maculopapular rash on both arms after 5 days of rFVIIa infusions.<sup>108</sup> One of the worries of using rFVIIa has been the risk of thromboembolic events. However, studies in hemophilia patients have shown that this risk is extremely low. There is some evidence, as well, that the initial treatment dose of rFVIIa could be increased as a one-time dose without thromboembolic complications. The bleeding episodes from the HTRS Registry

were treated with bolus IV doses ranging from  $<100\mu\text{g/kg}$  to a maximum of  $346\mu\text{g/kg}$ . None of the patients experienced a thromboembolic event. The doses used were divided into four groupings:  $<100\mu\text{g/kg}$ ,  $100$  to  $150\mu\text{g/kg}$ ,  $150$  to  $200\mu\text{g/kg}$ , and  $>200\mu\text{g/kg}$ . Bleeding stopped in 86%, 82%, 92%, and 93% for each of the respective groups of patients including the 23 patients who were under 18 years of age. In the adult groups, bleeding stopped in 100% of the patients treated with  $>200\mu\text{g/kg}$ .<sup>108</sup>

In those persons (usually infants and young children) who have severe hemophilia B and develop an inhibitor, roughly 40% to 50% will have severe allergic reactions (including anaphylactic shock) when infused with any Factor IX-containing product (plasma, plasma-derived Factor IX concentrates, or recombinant Factor IX).<sup>98</sup> Although a few such patients have been desensitized to Factor IX, for the majority of hemophilia B patients who have had severe allergic reactions to Factor-IX-containing products, rFVIIa is regarded as the treatment of choice.<sup>96</sup>

### Prophylaxis Using Recombinant Factor VIIa

Despite its short half-life, two recent studies demonstrated that a daily prophylactic dose of either  $90\mu\text{g/kg}$  or  $270\mu\text{g/kg}$  was effective in patients with inhibitors in decreasing the frequency of joint bleeding.<sup>102,111</sup>

### Staphylococcal Protein A Immunoabsorption of Inhibitor Antibodies

Staphylococcal protein A immunoabsorption columns (to rapidly reduce a very high-titer Factor VIII or Factor IX inhibitor) are widely available but not often used. In view of the availability of “bypassing” agents such as aPCC and rFVIIa, which work as well in patients with high-titer inhibitors, there is not often a need to rapidly reduce an inhibitor titer in a bleeding patient. The columns are, however, used on occasion in order to decrease an inhibitor titer in a patient in preparation for certain immune tolerance regimens, such as the Malmö regimen. The International Workshop on Immune Tolerance Induction consensus recommendations address the potential utility for immunoabsorption in patients with severe hemophilia B before immune tolerance in order to reduce the incidence of nephrotic syndrome.<sup>112</sup>

### Prevention of Inhibitor Development

The CANAL (Concerted Action on Neutralizing Antibodies in Severe Hemophilia A) study group studied children with severe hemophilia A who were started on regular prophylaxis at an early age (median age of 20 months). These children, who received treatment at least twice weekly, had a 60% reduction in the risk of developing inhibitors.<sup>113</sup> Other studies have confirmed these findings.<sup>114,115</sup> These studies infer that the institution of early prophylaxis may be a reasonable means for the prevention of inhibitor development and must be considered, particularly in patients at high risk for inhibitor development (positive family history, at-risk mutations).

### Attempts to Suppress or Eradicate Inhibitors: Immune Tolerance Regimens

In the 1970s, a demanding regimen for ITI, called the “Bonn protocol,” used very large and very frequent doses of both Factor VIII and aPCC (FEIBA) during a period of many months or even years.<sup>116</sup> In the 1980s other investigators began reporting success with modifications of the Bonn regimen that involve smaller doses of Factor VIII given at less frequent intervals.<sup>97</sup> Ewing et al<sup>117</sup> had considerable success with a regimen consisting of a daily infusion of Factor VIII of 50IU/kg, and others have reported<sup>118</sup> suppression of inhibitors in a high percentage of patients given Factor VIII at 25IU/kg on alternate days or two or three times a week. Other groups reported an enhancement of immune tolerance with the addition of intravenous  $\gamma$ -globulin, immunosuppressive agents, or both.<sup>119,120</sup>

Although these modifications of the Bonn regimen are costly and require good venous access and patient compliance (and a guaranteed supply of Factor VIII), it now seems that such approaches suppress many inhibitors completely and convert other high responders to low responders. Observations to date indicate that timing is extremely important in the use of ITI. The optimal time to start a regimen is when the inhibitor titer is  $<10$  BU; this is best accomplished by avoiding exposure to Factor VIII. It is important to watch the titer closely and initiate treatment promptly when the titer falls sufficiently. Of course, ITI may need to be started sooner if a 1- to 2-year period does not yield the desired titer levels or there is a life-threatening hemorrhage. The optimal dose and frequency for ITI varies between investigators and patients. Hemophiliacs with inhibitors can be divided into “good risk” and “poor risk” patients. The “good risk” patient has an inhibitor titer  $<10$  BU before the initiation of immune tolerance, has a maximum inhibitor level that never exceeded 199 BU, and is starting immune tolerance less than 5 years since the diagnosis of an inhibitor. The “poor risk” patient does not fall into those categories. There is some evidence that “good risk” patients may require less Factor VIII, but “poor risk” patients may require as much as 200IU/kg/day to maximize the efficacy of ITI.<sup>112</sup>

Although many physicians treating hemophiliacs now institute ITI in an attempt to suppress or eradicate Factor VIII inhibitors, the success rate has been only about 70%, and is poorer when immune tolerance is attempted in hemophilia B patients with inhibitors. The North American Immune Tolerance Registry data suggested only a 36% success rate in hemophilia B patients; however, patients with allergic reactions were over-represented.<sup>121</sup> The success rate is hindered by the development of nephrotic syndrome, which has been reported in 13 patients internationally. This generally occurs 8 to 9 months after the start of ITI, and its etiology remains unclear. Although the nephrotic syndrome generally resolves with the avoidance of Factor IX products, this complication often mitigates further ITI.<sup>122</sup>

### Gene Therapy

Gene therapy replaces the missing factor or protein with a nucleic acid rather than the protein itself. The goal would be to transfect certain targeted tissues with the nucleic acid to replace

the mutated area of the genome. Hence, the targeted tissue would produce normal protein either fully or partially correcting the factor deficiency. Hemophilia has a favorable combination of features for a positive response to gene replacement therapy. A reduced-intensity stem cell transplant in mice with stem cells transduced with the BDD porcine Factor VIII has been effective in decreasing clot times and increasing Factor VIII levels.<sup>123</sup> A few dog models have been used successfully in transgene expression. The targeted tissues were skeletal muscle and liver/hepatocytes. The problem of immune reaction to the transgene product exists and may necessitate approximately 6 months of immune suppression. Vector safety is another important consideration in transporting the transgene product to the recipient.<sup>124</sup> A few gene therapy trials in humans have now been completed and the preliminary data are moderately encouraging; however, the results are far from satisfactory with regard to safety and efficacy.<sup>125</sup> In terms of efficacy, the plasma levels of Factor VIII or Factor IX reached so far are insufficient to free patients from the need for infusion of exogenous factors. No inhibitor developed in gene replacement trials, but this risk is still of concern especially in previously untreated patients. Additionally, small amounts of the viral genome have been detected in the semen of a few patients, suggesting that the risk of germline integration and passage from the vector to descendants cannot be ruled out.<sup>19,125</sup>

## von Willebrand Disease

von Willebrand disease is characterized by mucous membrane bleeding, excessive bruising, and excessive bleeding during and after surgery or invasive dental procedures. It is the most common of the hereditary coagulation disorders. The incidence of vWD in the United States is estimated to be between 0.82% and 1.6% of the population. Clinical severity varies greatly, and many affected individuals have minimal symptoms unless challenged by surgery. A number of subtypes of vWD have been described; the more common of these are discussed below (Types 1, 2, and 3).

The basic defect is in vWF, a large multimeric plasma glycoprotein (GP) that supports the adhesion of platelets to the vascular subendothelium. It is normally present in plasma in multimers of up to 20 million daltons. The highest-molecular-weight multimers are the most important hemostatically for platelet adhesion and connective tissue binding. vWF circulates as a complex with Factor VIII and protects Factor VIII from rapid proteolytic degradation, as well as transporting it to sites of active hemostasis.

vWF is synthesized in endothelial cells and megakaryocytes and is stored in organelles (in Weibel-Palade bodies of endothelial cells and in the alpha granules of platelets). The gene for vWF is located on the short arm of chromosome 12.<sup>126</sup> vWF levels are influenced by ABO blood group,<sup>127</sup> with the lowest levels being found in persons of blood group O and the highest in those with blood group AB. The A and B alleles code for A and B glycosyltransferase, which converts the H antigen into A and B blood groups. Blood group O individuals lack this enzyme and

continue to express the H antigen. H-antigen-rich blood groups have significantly lower vWF and Factor VIII.<sup>128</sup> Hormonal changes accompanying pregnancy increase the levels of vWF, Factor VIII, and fibrinogen.

von Willebrand factor is a plasma glycoprotein composed of multimeric subunits. It is the high-molecular-weight (HMW) subunits that mediate platelet adhesion during vessel injury. vWF also binds to Factor VIII in the circulation and stabilizes its presence there. In the absence of vWF (Type 3 vWD), Factor VIII levels are also very low. Once released from the Weibel-Palade bodies or alpha granules, the multimers can interact with platelets, connective tissue, or other cells, but the larger subunits may also be cleaved by the ADAMTS13 metalloprotease into smaller multimers. These cleavage products are seen on an agarose gel when a von Willebrand multimer analysis is ordered. A mutation involved in multimer assembly and function results in Type 2 vWD.<sup>126</sup>

von Willebrand disease results from a quantitative or qualitative deficiency of vWF. If vWD is suspected, a battery of tests should be performed. Screening tests such as the aPTT and bleeding time may or may not be abnormal, especially in persons with mild vWD. More specific tests include quantitation of vWF:Ag by an immunoelectrophoretic assay (the Laurell technique) or by an enzyme-linked immunosorbent assay, a ristocetin cofactor (RCoF) assay as a measure of vWF activity, a Factor VIII assay, and multimeric analysis of vWF using sodium dodecyl sulfate agarose gel electrophoresis.

## Classification of von Willebrand Disease

A revised classification from an international subcommittee, intended to reflect the research on the function and fate of vWF and differences in pathophysiologic mechanisms that lead to particular vWD phenotypes, was recently published.<sup>126</sup> Type 1 vWD is by far the most common of the types of vWD, accounting for at least 80% of cases. The Type 2 variants account for 10% to 15%, whereas Type 3 is relatively rare (Table 28-3).

Type 1 vWD is caused by autosomal dominant heterozygous mutations. The diagnosis of vWD Type 1 is difficult because of significant variations in vWF levels as discussed above. Bleeding symptoms also vary widely with vWF:Ag ranges of 30 to 50 IU/dL<sup>-1</sup>. Linkage to a vWF gene mutation has been shown to be only 0.4 to 0.5 in patients with vWF:Ag levels >30 IU/dL<sup>-1</sup>.<sup>126</sup> The diagnosis of Type 1 vWD is characterized by a prolonged closure time on the platelet function analyzer and proportionately low levels of vWF:Ag, vWF activity, and Factor VIII. Therefore, a vWF:RCoF/vWF:Ag ratio should be proportionate (0.7-1.2) for Type 1 vWD.<sup>126,129</sup> Multimeric analysis of the patient's vWF reveals that all sizes of vWF multimers are present. In Type 1 vWD, vWF levels are subnormal, but the vWF produced is structurally and functionally normal.

Type 2 variants are characterized by qualitative defects in vWF.<sup>126</sup> Type 2A results either from decreased production or from accelerated clearance of the hemostatically important

**Table 28-3.** Laboratory Diagnosis of von Willebrand Disease and Suggested Treatment Options\*

| Condition | vWF:RCoF (IU/dL) | vWF:Ag (IU/dL)       | Factor VIII     | vWF:RCoF/vWF:Ag  | Therapeutic Options                 |
|-----------|------------------|----------------------|-----------------|------------------|-------------------------------------|
| Type 1    | <30 <sup>†</sup> | <30 <sup>†</sup>     | ↓ or Normal     | >0.5-0.7         | DDAVP/Stimate <sup>‡</sup>          |
| Type 2A   | <30 <sup>†</sup> | <30-50 <sup>‡§</sup> | ↓ or Normal     | <0.5-0.7         | DDAVP; vWF-Factor VIII concentrates |
| Type 2B   | <30 <sup>†</sup> | <30-50 <sup>‡§</sup> | ↓ or Normal     | Usually <0.5-0.7 | vWF-Factor VIII                     |
| Type 2M   | <30 <sup>†</sup> | <30-50 <sup>‡§</sup> | ↓ or Normal     | <0.5-0.7         | vWF-Factor VIII                     |
| Type 2N   | 30-200           | 30-50                | ↓↓              | >0.5-0.7         | vWF-Factor VIII                     |
| Type 3    | <3               | <3                   | ↓↓↓ (<10 IU/dL) | Not applicable   | vWF-Factor VIII                     |
| "Low vWF" | 30-50            | 30-50                | Normal          | >0.5-0.7         | Case-by-case need                   |
| Normal    | 50-200           | 50-200               | Normal          | >0.5-0.7         |                                     |

\*Adapted with permission from Sadler et al.<sup>126</sup>

<sup>†</sup><30 IU/dL is designated as the level for a definitive diagnosis of vWD; there are some patients with Type 1 or Type 2 vWD who have levels of vWF:RCoF and/or vWF:Ag of 30-50 IU/dL.

<sup>‡</sup>In nonresponsive patients, use a vWF-Factor VIII concentrate.

<sup>§</sup>The vWF:Ag in the majority of individuals with Type 2A, 2B, or 2M vWD is <50 IU/dL.

↓ Refers to a decrease in the test result compared to the laboratory reference range.

vWF:RCoF = von Willebrand factor:ristocetin cofactor; vWF:Ag = von Willebrand factor antigen; DDAVP = desmopressin.

HMW vWF multimers. The loss of HMW multimers causes a decrease in platelet-vWF interactions as expressed in a decreased vWF:RCoF assay result. Loss of HMW multimers can also cause a decrease in connective tissue-vWF interactions as measured in decreased vWF:collagen binding (vWF:CB) assay results. Patients may have normal or only slightly reduced vWF and Factor VIII levels but may have a loss in the intermediate and HMW multimers of vWF in plasma. Affected individuals usually have a mild-to-moderate bleeding tendency and generally have a poor response to DDAVP.<sup>126,129</sup>

In vWD Type 2B, mutations in the A1 domain results in a heightened affinity of vWF binding to platelet glycoprotein 1b (GP1b). Affected individuals often have mild thrombocytopenia caused by in-vivo platelet aggregation, and on ex-vivo testing, their platelets aggregate with very low concentrations of ristocetin (enhanced ristocetin-induced platelet aggregation). Upon secretion, the HMW multimers in vWD Type 2B bind spontaneously to platelets and are then cleaved by ADAMTS13 for excretion. The small multimers do not enhance platelet adhesion or connective tissue binding, resulting in decreases in vWF:RCoF and vWF:CB. Another disorder caused by a mutation on the platelet GP1b causes a similar phenotype with increased binding affinity of the platelet for vWF. This is called pseudo-vWD.

von Willebrand disease Type 2M variants have decreased affinity of vWF for platelets. This results in an increase in HMW multimers on the agarose gel testing. These multimers have a decreased activity in platelet adhesion and decreased cleavage by ADAMTS13. Similar to Type 2B, most patients with Type 2M have a mutation in the vWF A1 domain responsible for binding the GP1b.

von Willebrand disease Type 2N is an uncommon but interesting variant. It is characterized by a mutation in vWF that prevents the binding of Factor VIII but does not interfere with

platelet adhesion. Thus, uncomplexed Factor VIII is rapidly degraded in the circulation, resulting in low levels of Factor VIII, mimicking hemophilia A.<sup>126</sup>

In vWD Type 3, patients have a severe bleeding tendency and very low levels of vWF and Factor VIII. Type 3 is inherited in an autosomal recessive pattern and has levels of vWF:Ag <1 to 5 U/dL. Although the vWF:Ag levels of Type 3 can approach those of severe Type 1, the two types of vWD are almost always clinically distinguishable. In addition to severe mucous membrane bleeding, Type 3 patients may bleed into joints and soft tissues. Type 3 patients are not responsive to desmopressin, whereas type 1 patients generally are responsive (see below). Most cases of vWD Type 3 are caused by deletions, nonsense, missense, and frameshift mutations in the vWF gene.<sup>126,129</sup>

### Treatment of von Willebrand Disease

Treatment is determined by the type of vWD (Table 28-3). The goals of therapy are to increase both vWF and Factor VIII levels, which predict clinical response. Generally, raising the vWF and Factor VIII levels into the normal range (above 50%) is sufficient to alleviate the bleeding manifestations of vWD. However, a qualitative defect (Type 2 vWD) may also require completely replacing the patient's vWF with normal vWF to give a clinical response.

For persons with Type 1 vWD, the treatment of choice is DDAVP.<sup>74,76,129,130</sup> When given intravenously in a dosage of 0.3 µg/kg diluted in 50 mL saline over 30 minutes, the drug will effect a rapid three- to fivefold increase in vWF activity, vWF:Ag, and Factor VIII levels in most patients with Type 1 vWD and some with Type 2A. Generally, the increase in vWF and Factor VIII levels will last for 6 to 8 hours. It is important to give a patient with vWD a test dose of DDAVP to confirm responsiveness and to determine the magnitude of the response.

Although the magnitude of response varies among individuals, the response in a given individual is generally consistent over time (ie, threefold increases in vWF activity and vWF:Ag on one occasion are likely to be duplicated during retreatment at a later date). The dose can generally be repeated every 12 to 24 hours with efficacy. Most patients treated repeatedly over a short period do become less responsive, a phenomenon called tachyphylaxis.<sup>76</sup> Because the effectiveness of DDAVP is attributable to the rapid release of vWF from storage sites, DDAVP is ineffective in patients with Type 3 vWD, who have nothing in storage to be released. Most clinicians consider DDAVP to be contraindicated in Type 2B, because the release of the functionally abnormal vWF causes a worsening of thrombocytopenia and is hemostatically ineffective.<sup>76</sup> Although desmopressin is generally given intravenously to hospitalized patients, the highly concentrated intranasal spray formulation is ideal for home use. It provides excellent bioavailability of the drug and effects an increase of vWF similar to that obtained with an intravenous dose of 0.3 µg/kg DDAVP. The compression metered spray pump delivers 0.1 mL (150 µg) of solution per spray. The dose for children and adolescents is one spray; for adults a spray is given in each nostril. This highly concentrated intranasal spray is particularly useful for prophylaxis before invasive dentistry or other minor surgery.<sup>131</sup>

The side effects of DDAVP are generally well tolerated. Facial flushing, mild tachycardia, and headaches are often transient and mild. DDAVP does have an antidiuretic effect and rarely can cause hyponatremia, leading to seizures. This effect has been seen most often in the use of DDAVP in small children. All patients using the drug at home must be counseled to not give repeat doses within 24 hours without consulting a physician. Thrombosis has been reported in DDAVP treatment of diseases other than vWD, and in one patient with concurrent vWD and thrombophilia.<sup>76</sup>

Replacement therapy with a plasma-derived intermediate-purity Factor VIII concentrate rich in vWF is still needed in certain situations. For patients with vWD Type 1 or Type 2A whose responses to DDAVP are not sufficient (eg, for major surgery), a clotting factor concentrate containing the HMW multimers of vWF should be given.<sup>74,130</sup> For persons with vWD Types 2B and 3, concentrates rich in the hemostatically important HMW multimers of vWF should be given for the treatment of serious bleeding episodes or for surgical prophylaxis.

The optimal replacement therapy for vWD corrects both the vWF defect (quantitatively and qualitatively) and the Factor VIII concentration. Because most commercial Factor VIII concentrates do not contain the hemostatically important HMW multimers of vWF, several investigators have conducted comparative studies of various products in patients with severe vWD. Humate-P (CSL Behring) was found to contain the highest amount of HMW multimers and therefore to be most effective for patients with vWD.<sup>74,130,132</sup> This pasteurized concentrate is thought to be safer than cryoprecipitate, which is not virus-inactivated. For the treatment of severe bleeding or for major surgeries in patients with Type 3 vWD, some studies recommend a bolus dose of 60 to 80 IU vWF:RCoF/kg<sup>-1</sup> followed by 40 to 60

vWF:RCoF/kg<sup>-1</sup> every 8 to 12 hours for a few days then spaced out to daily dosing as needed for control of bleeding. A multicenter, retrospective study<sup>132</sup> showed an average daily use of 80 IU vWF:RCoF/kg<sup>-1</sup> per day for surgery coverage. The efficacy was 97% in these patients. The on-demand treatment of bleeding averaged 72 IU vWF:RCoF/kg<sup>-1</sup>/day of Humate P with an efficacy of 95% in these patients. A prospective, multicenter open-label cohort study<sup>133</sup> was conducted in patients with hereditary vWD (acquired vWD patients excluded) and a history of abnormal bleeding. The median vWF:RCoF dose after individual pharmacokinetic testing was 62.4 IU/kg<sup>-1</sup> with full control (analogous to expected bleeding in a normal patient) of intraoperative bleeding in 96.3% of the patients.<sup>133</sup>

An interesting use of DDAVP and Humate P occurred in a hemophilia treatment center in Germany. Two patients had severe hemophilia A with Type 1 vWD, one patient had severe hemophilia B with Type 1 vWD, and two patients had severe hemophilia B with Type 2 vWD. All five patients experienced more mucosal bleeding than would be expected with a diagnosis of hemophilia alone. The two of these patients with severe hemophilia B and Type 2 vWD had very difficult-to-treat bleeding with recombinant replacement of Factor IX alone. Even with prophylaxis, breakthrough bleeding was a problem. These patients responded to concurrent infusions of BeneFix and Humate P or DDAVP.<sup>134</sup>

Other agents that may be useful include the antifibrinolytic drugs, ε-aminocaproic acid and tranexamic acid. For the treatment of minor mucosal bleeding, particularly in patients with mild vWD, an antifibrinolytic drug may suffice. Antifibrinolytic agents also should be used in conjunction with desmopressin or Humate-P for invasive dental procedures, tonsillectomy, or other bleeding in the oral cavity. As is true for all coagulopathies, persons with vWD should avoid aspirin, all aspirin-containing compounds, and other drugs that interfere with platelet function.

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## Other Inherited Disorders of Coagulation

Rare hereditary deficiencies of all the other known coagulation factors have been described, and are heterogeneous. Thus, not all affected families with a particular factor deficiency have the same bleeding tendencies. Because these disorders are inherited in an autosomal recessive pattern, patients who are homozygotes or compound heterozygotes tend to bleed more. Because no specific clotting factor concentrates are licensed and available in the United States for use in these relatively rare deficiency states, the treatment of choice for bleeding in most of them is still PCCs, plasma, or cryoprecipitate. In general, the level required to achieve hemostasis is relatively low and thus can be attained by plasma in a dosage of 10 mL/kg. Once hemostasis has been achieved, continued treatment may not be necessary.<sup>135</sup> However, plasma (solvent/detergent-treated FFP or donor-retested FFP) and cryoprecipitates have the disadvantages of potential viral contamination, volume overload, and allergic reactions.

Bleeding in persons with congenital afibrinogenemia or hypofibrinogenemia should be treated with cryoprecipitate in a dosage of four bags/10 kg of body weight. Cryoprecipitate contains approximately 200 mg of fibrinogen per bag, or “unit.” (Because of the long half-life of fibrinogen, replacement therapy is generally given every 3 to 4 days.) Although seldom necessary, additional doses of two bags/10 kg can be given every 3 to 4 days. In Europe a specific fibrinogen concentrate (treated to be virus-free) is available.

In rare hereditary factor deficiencies (ie, Factor II, V, or X deficiencies), plasma or a PCC can be used. For serious, life-threatening bleeding, PCCs should be used to achieve higher levels of the missing factor. Recombinant Factor VIIa is the treatment of choice for congenital Factor VII deficiency, for on-demand treatment and prophylaxis. The current recommended dose for surgical or invasive procedures is 15 to 30 µg/kg every 4 to 6 hours.<sup>100</sup> Deficiencies of the “contact factors,” Factor XII, prekallikrein, and HMW kininogen, are rarely associated with clinical bleeding and thus do not require treatment.

The hemorrhagic tendency in Factor XI deficiency is usually mild and independent of the Factor XI levels. On occasion excessive bruising, epistaxis, and postoperative bleeding have been observed. One study<sup>136</sup> found that bleeding tendency in tissues with fibrinolytic activity (ie, oral mucosa, tonsils, nose) was increased 49% to 67%, while the tendency to bleed in other tissues was reduced to 1.5% to 40% irrespective of Factor XI deficiency genotype. Therefore, in patients with Factor XI undergoing a procedure in areas of fibrinolytic activity, an antifibrinolytic agent with on-demand replacement may be sufficient. Patients undergoing other surgeries or procedures such as circumcision or appendectomy may require on-demand therapy only for intra- or postoperative bleeding.<sup>135</sup> FFP infused at the rate of 10 to 20 mL/kg/day usually provides sufficient Factor XI to maintain hemostasis. The aPTT, the specific assay for Factor XI, or both should be used to monitor treatment.

Congenital deficiency of Factor XIII (fibrin-stabilizing factor) is a rare disorder characterized by delayed umbilical bleeding in neonates, subcutaneous bleeding, muscle hematomas, bleeding after surgery, hemarthroses, and ICH.<sup>136</sup> It is also characterized by a lifelong bleeding tendency and impaired wound healing. Only homozygous individuals with no detectable Factor XIII activity have bleeding manifestations. Factor XIII circulates in the plasma with the A and B subunit in an A<sub>2</sub>B<sub>2</sub> tetramer. The patients with most severe disease have a deficiency in the A subunit. The prothrombin time, aPTT, and thrombin time are normal in Factor XIII deficiency. The diagnosis used to be made exclusively by testing for clot solubility in 5 mol urea, but currently both Factor XIII assays and assays for the A- and B-subunit antigens are available. Unfortunately, the clot solubility testing is useful only for patients with zero Factor XIII activity, but bleeding may still be a problem at higher levels. Additionally, it is difficult to interpret the Factor XIII levels at low levels; the accuracy of this test at levels <10% is questionable. There is no correlation between bleeding and Factor XIII levels.<sup>137</sup> Fibrogammin

P (CSL Behring), although not licensed in the United States, is available on a clinical trial basis for patients with severe Factor XIII deficiency.<sup>74</sup> Because the half-life of Factor XIII is long, prophylactic therapy with Factor XIII concentrates can be given with a monthly dosage of 250 IU in children and 500 IU in adults.<sup>138</sup> If it is not readily available for some reason, a single infusion of plasma of 5 to 10 mL/kg usually will provide effective therapy for bleeding episodes.

## Hypercoagulable States

### Congenital and Acquired Thrombophilia

Thrombophilia refers to a blood condition, whether acquired or inherited, with an increased propensity to develop thrombosis. Acquired thrombophilia is seen in autoimmune and infectious or postinfectious states. Inherited thrombophilia indicates a congenital deficiency of an antifibrinolytic agent or a genetic mutation in one of the clotting factors leading to an increase risk of thrombosis. An example of acquired thrombophilia is antithrombin deficiency. Antithrombin deficiency can be seen in nephrotic syndrome, protein-losing enteropathy, and as a side effect of the commonly used chemotherapeutic agent, L-asparaginase. Additionally, protein C and S deficiencies can be seen as a consumptive process in patients with severe sepsis and DIC.<sup>139</sup> Protein C deficiency, in particular, has been associated with an increased risk for mortality in sepsis and protein C replacement is being investigated in the treatment of meningococcal-induced purpura fulminans.<sup>140</sup> Antiphospholipid antibodies including lupus anticoagulant often occur after infections and are self-limited. Detection of antiphospholipid antibodies in the serum for 6 weeks or more connotes antiphospholipid antibody syndrome (APAS) and is more commonly seen in adolescents. APAS may be a result of lupus or other autoimmune disorders (Table 28-4).<sup>139</sup>

Genetic mutations cause hypercoagulability by decreasing the amount or function of various proteins responsible for the regulation of coagulation. These proteins include antithrombin, protein C, protein S, plasminogen, and fibrinogen. Point mutations in genes encoding prothrombin and Factor V, such as prothrombin 20210 and Factor V Leiden, represent a genetic risk factor for thrombophilia. The prothrombin 20210 mutation

**Table 28-4.** Risk Factors for Thrombosis in Children<sup>141</sup>

#### Temporary Risk Factors

- Indwelling catheters
- Surgery
- Infection

#### Ongoing Risk Factors

- Thrombophilia—genetic or acquired
- Malignancy and chemotherapy
- Inflammatory diseases
- Prosthetic heart valves
- Sickle cell anemia

causes a 15% to 30% increase in the production of the procoagulant, prothrombin. Factor V Leiden is a mutation that causes resistance of Factor V to physiologic downregulation by activated protein C. Other genetic hypercoagulable states include hyperhomocysteinemia and platelet membrane glycoprotein defects. In a recent study<sup>142</sup> involving children with stroke, laboratory testing was performed to look for a prothrombotic condition in 338 of the study participants. Of these 338 children, 64% percent had laboratory results consistent with a prothrombotic condition, 24% had a Factor V Leiden mutation, and another 12% had antiphospholipid antibodies detected.<sup>142</sup>

## Venous Thromboembolism in Infants and Children

### Incidence and Risk Factors

Venous thromboembolism (VTE) is relatively rare in the pediatric population. A multicenter Canadian VTE Registry trial<sup>143</sup> reported an incidence in the general pediatric population of 7 per million children or 5.3 per 10,000 hospital admissions in children. The risk for VTE is greatest in the neonatal and adolescent age groups with indwelling catheters representing the most common risk factor found in 33% of children with VTE. It is for this reason that VTEs in children are more commonly seen in the upper venous system, contrary to the location of VTEs in adults.<sup>144</sup> A study<sup>145</sup> that investigated the incidence of VTE in children with acute lymphoblastic leukemia treated with L-asparaginase, also studied risk factors for VTE in children with indwelling catheters. Left-sided catheters, particularly in the subclavian vein, inserted by percutaneous rather than venous cut-down technique were at greatest risk of catheter-associated VTE.<sup>145</sup> The current recommendations from the American College of Chest Physicians do not support primary anticoagulation prophylaxis in children with indwelling catheters.<sup>146</sup>

Only 3.6% of children on the Canadian Registry with VTE had thrombosis with no predisposing condition.<sup>143</sup> All others had at least one predisposing factor including catheters, cancer, congenital heart disease, infection, trauma, obesity, nephrotic syndrome, surgery, lupus, sickle cell disease, and liver failure. Twelve children had a prothrombotic condition—six had protein C deficiency and six had protein S deficiency. The contribution of a prothrombotic condition is difficult to assess from these data, because only 33% (45 of 137) of the children with VTE underwent testing for a prothrombotic condition.

Umbilical venous catheters can also be a source for clot formation. These clots often go undetected and occur in the inferior vena cava and portal vein. Clots from umbilical venous catheters can cause lobar atrophy or portal hypertension that may be discovered later in life. An incidence of 3.6 per 1000 neonates admitted to the neonatal intensive care unit at Toronto's Hospital for Sick Children has been reported.<sup>147</sup> Anticoagulation did not appear to affect the development of portal hypertension or lobar atrophy.

In a pediatric stroke trial,<sup>142</sup> 36% of the 1065 children studied had cerebral sinovenous thrombosis (CSVT) and 54% had arterial ischemic strokes (AIS). Of the children with CSVT, 39% had identifiable risk factors for VTE. The older infants and children had a statistically higher number of identifiable risk factors than the neonates. Systemic disease was an identifiable risk factor for children with CSVT. Systemic diseases included genetic syndromes, malignancy, chronic infection, hypertension, and chronic prenatal conditions. Of the children with genetic syndromes, 38.5% had Down syndrome. Other genetic syndromes were not specified but were associated with congenital heart disease or other chronic medical conditions. Acute infections were also reported more commonly in children with CSVT than those with AIS.

### Diagnosis and Treatment

The diagnostic evaluation for VTE is dependent upon the location of the suspected clot and the clinical index of suspicion. Clots related to umbilical venous catheters are best diagnosed with ultrasound and/or venography. Clots related to indwelling central venous catheters often develop in the upper venous system. The initial evaluation would include a color Doppler ultrasound, but often venography and an echocardiogram are necessary to identify the suspected clot. Doppler ultrasound carried a sensitivity of only 37% in one study,<sup>145</sup> but it has the advantage of a noninvasive evaluation. The sensitivity of bilateral venography was found to be 79%, but venography is a more invasive method of detection. Venography was the most sensitive for the central upper venous system, whereas Doppler appeared superior in the diagnosis of jugular venous clots. The diagnosis of VTE in the upper venous system should encompass Doppler sonography, echocardiography, and the more invasive alternative, venography, as needed.<sup>148</sup>

Data are limited on the optimal treatment of VTE in children. Evidence-based evaluation and management of pediatric thrombosis and thrombophilia is in its infancy. Most of the information available today is based largely on small case series or extrapolated from adult recommendations.<sup>141</sup> The agents currently available for antithrombotic therapy in children include UFH, low-molecular-weight (LMW) heparin, warfarin, and tissue plasminogen activator (tPA). UFH is an effective drug, but has a short half-life of 25 minutes in neonates (to 1 hour in adults).<sup>149</sup> LMW heparins are being used increasingly for initial therapy for acute thrombosis in children. In one study,<sup>150</sup> 53% of 190 children treated with enoxaparin for thromboembolism had complete resolution of the thrombus. Children with arterial clots and clots that were not occlusive at diagnosis were more likely to have complete resolution of their clots after taking enoxaparin. Forty-eight percent of the children with VTE had complete resolution of the thrombus; this is not appreciably different from the adult data of VTEs treated with LMW heparins. Age at diagnosis, location of the thromboembolus (excluding arterial vs venous), and initial treatment with fractionated vs UFH did not appreciably affect the complete

resolution of the clots. The mean dose of enoxaparin also did not affect the complete resolution of the clot.<sup>150</sup>

LMW heparins have many advantages over UFH and other anticoagulants in children, and they are rapidly becoming the treatment of choice for children with hypercoagulability and clots. The advantages of treatment with LMW heparin include: effectiveness equal to UFH, a lower frequency of bleeding complications, decreased incidence of heparin-induced thrombocytopenia, minimal monitoring required (the pharmacokinetics of LMW heparin are more predictable than those of UFH), decreased amount of drug-to-drug interactions, and possibly a decreased risk of osteoporosis with long-term treatment relative to UFH. The current recommended dose of LMW heparin depends on the anti-Factor Xa level in a sample drawn 4 to 6 hours after a subcutaneous dose of LMW heparin. The targeted therapeutic anti-Factor Xa level is generally 0.5 to 1 U/mL. Neonates and infants less than 2 to 3 months of age or 5 kg of weight generally require a higher dose per kilogram to maintain therapeutic anti-Factor Xa levels. Because of increased clearance in children, LMW heparins (especially enoxaparin) are administered subcutaneously twice a day.<sup>146</sup> Schobess et al<sup>151</sup> recently evaluated 80 children over 3 months of age with DVT in an open-label pilot randomized safety study, using once-a-day enoxaparin administration, with a target 4-hour anti-Factor Xa activity between 0.5 and 0.8 IU/mL. The children were stratified to receive either once daily or twice daily doses. The mean duration of treatment was 5 months, with a median follow-up of 24 months. The authors found no significant differences between the two groups with regard to the study endpoints, postthrombotic syndrome, rethrombosis, bleeding, or therapy-related death.<sup>151</sup>

Systemic or local thrombolysis should be strongly considered in children with “high-risk” clots that present within 2 weeks of symptomatic onset. Low-dose (0.03–0.06 mg/kg/hour) systemic infusions of tPA for 12 to 96 hours have been shown to be effective in children.<sup>152</sup> Warfarin, despite being an oral agent, is difficult to administer in children and requires frequent monitoring with dose adjustments.<sup>153</sup> Children with thrombophilia do not always require lifelong anticoagulation even if a genetic cause for hypercoagulability is isolated. A single thrombophilic gene mutation can certainly cause an increased risk of thrombosis in childhood; however, it is not necessarily implicated in a worse outcome or greater risk of recurrence.<sup>154</sup> For instance, no increase in DVT-related pulmonary embolus was seen in otherwise-healthy children diagnosed with thrombophilia.<sup>155</sup> An elevated D-dimer in the absence of infection or chronic inflammation can be indicative of excessive thrombin generation and suggest a need for prolonged anticoagulation. In addition, a recurrence of thrombus could be a strong indication for prolonged or possibly lifelong anticoagulation with or without an isolated genetic thrombophilia.<sup>139</sup> Because prospective randomized studies in children are lacking, one strategy to select optimal antithrombotic therapy is to tailor treatment based upon assessed risk for an unfavorable clot outcome. Manco-Johnson,

in a review of antithrombotic therapy, suggested using patient-specific and thrombus-specific characteristics to stratify the likelihood of a poor outcome.<sup>141</sup> Thus, children without underlying thrombophilia in whom a transient triggering event has resolved are classified as “low risk” while those with at least three thrombophilic traits, or those with persistence of antiphospholipid antibodies who at the time of presentation have an occlusive DVT, a Factor VIII level >150 U/dL, and a D-dimer level >500 ng/mL are considered “high risk” (Table 28-5).

### Complications

A Canadian study reports a recurrence rate of approximately 8%.<sup>144</sup> The recurrence rate is higher with increasing age of the child at diagnosis. Mortality is uncommon but occurs in 2.2% of children with deep vein thrombosis (DVT)/pulmonary embolus.<sup>156</sup>

Postthrombotic syndrome (PTS), also referred to as postphlebotic syndrome, is an important consequence of DVT. PTS describes limb pain, swelling, development of visible collateral circulation, and chronic venous insufficiency. PTS can often be severe enough to limit the child’s activity. The incidence of PTS following childhood DVT is difficult to assess and estimated between 10% and 63% depending on the study.<sup>157,158</sup> It is felt to be underreported and underdiagnosed in childhood. The wide variation in reporting has been due in part to a lack of standardization among assessment tools used to evaluate this problem in children. A pediatric scale for PTS has recently been validated in children.<sup>159</sup> PTS has been reported to increase in patients with elevated Factor VIII and D-dimer levels. A thrombolytic treatment study in high-risk pediatric DVT was shown to significantly

**Table 28-5.** Risk Assessment for Poor Outcome from a Thrombotic Event in Children<sup>141</sup>

#### Patient Characteristics

- Low-risk features
  - Transient triggering event
  - No thrombophilic trait
- Standard risk features
  - Less than three thrombophilic traits
  - Factor VIII <150 U/dL and D-dimer <500 ng/mL at the time of presentation
- High-risk features
  - At least three thrombophilic traits
  - Persistent antiphospholipid antibody >6 weeks
  - Factor VIII >150 U/dL and D-dimer >500 ng/mL at the time of presentation

#### Thrombus Characteristics

- Low-risk features
  - Thrombus resolved within 6 weeks
  - Nonocclusive thrombus
- High-risk features
  - Occlusive deep venous thrombosis
  - Persistence of thrombus at 6 weeks



decrease the incidence of PTS in children with “high-risk DVT.”<sup>160</sup> High risk was defined as acute lower extremity DVT and measured Factor VIII level  $\geq 150$  U/dL and/or D-dimer  $\geq 500$  ng/mL. The patients were stratified to receive thrombolytic therapy with an initial low-dose infusion of tPA. The tPA was continued until radiographic studies showed complete resolution of the thrombus. All of the patients received anticoagulation with either UFH to maintain a plasma anti-Factor Xa level of 0.3 to 0.7 U/mL or enoxaparin to maintain a level of 0.5 to 1.2 U/mL for at least 7 days. The long-term anticoagulation was accomplished with enoxaparin or warfarin for at least 3 months from diagnosis.

Pulmonary embolus is a well-known complication of DVT in adults, but it is likely that many cases in children go unrecognized. Pulmonary embolus occurred in 16% or 39 children with central venous catheters in the Canadian Pediatric Thrombophilia Registry.<sup>161</sup> Eleven of these children had isolated episodes, and the remaining 28 developed pulmonary embolus after catheter-related DVTs. Thirteen of the DVTs were located in the lower venous system, and 15 DVTs were located in the upper venous system. There is no single “best” imaging technique for pulmonary embolus. A positive helical computed tomography scan confirms the diagnosis, whereas a normal ventilation-perfusion scan rules this out.<sup>141</sup>

## Arterial Thromboembolism in Infants and Children

### Incidence and Risk Factors

Arterial thromboembolism occurs most often in the setting of catheter-related endothelial trauma.<sup>139</sup> In the neonatal period, umbilical artery catheterization with vascular injury precipitates the thrombus. After the neonatal period, peripheral arterial catheterization and cardiac catheterization are the most common precipitating events.<sup>144</sup> Arterial thromboembolism in childhood is rare without a catheter-related event, but some predisposing risk factors have been identified. Familial hyperlipidemia, hyperhomocysteinemia, Takayasu arteritis, Kawasaki disease, congenital arterial malformations, and congenital heart disease all serve as predisposing risk factors for arterial thromboembolism. In addition, children who test positive for lupus anticoagulant are at risk for venous and arterial thrombosis.

Arterial ischemic stroke is rare in neonates, with an estimated annual incidence of 12 per 100,000 in neonates and 0.5 to 6 per 100,000 in children older than 1 year.<sup>144</sup> In the pediatric stroke study,<sup>142</sup> 675 children had AIS; of these children, 46% had identifiable risk factors for arterial thromboembolism, and 60% had vasculopathy. Cardiac disorders were found in 11% and other chronic disease occurred in 24%. Ten of the 115 children with cardiac disease and AIS were older children with a patent foramen ovale. Approximately 65% of the children, equal numbers for CSVT and AIS, tested positive for a prothrombotic disorder.

### Diagnosis and Treatment

The diagnostic workup for arterial thromboembolism depends on the suspected location of the clot or ischemia. Angiography

is helpful for peripheral catheter-related arterial thromboembolism. The diagnosis of AIS generally is confirmed with an MRI (79% of the cases in the stroke study). Magnetic resonance angiography can be helpful for small or recurrent clots that are not well visualized on a standard MRI. Recurrent embolic AIS could occur from a cardiac defect or cardiac vegetations, which should be ruled out with contrast echocardiography.

Children who develop Kawasaki disease should be treated with aspirin therapy and IV gammaglobulin replacement within 10 days of onset. The aspirin doses should begin at 80 to 100 mg/kg/day during the initial or acute phase, up to 14 days from the onset of symptoms. Aspirin should be continued in lower doses, 3 to 5 mg/kg/day for at least 7 weeks more.<sup>146</sup>

As discussed previously, children with arterial thrombosis have been found to be more responsive to treatment with LMW heparin than children with venous thrombosis, particularly in a nonocclusive clot. LMW heparin has also been used acutely to treat children with AIS, particularly in children found to have vasculopathies, thrombophilia, or antiphospholipid antibodies.<sup>142</sup> Neonatal strokes are generally not treated and generally do not recur. Randomized clinical trials are necessary to identify the thromboembolic states that mandate treatment and determine the optimal treatments when treatment is needed.

## Summary

Hemophilia A (Factor VIII deficiency) and hemophilia B (Factor IX deficiency) are clinically indistinguishable, sex-linked coagulopathies that affect approximately 20,000 persons in the United States. Hemophilia A is the more common of the two and accounts for 80% of the cases. In the past, the treatment of hemophilia with plasma-derived products resulted in extremely high rates of hepatitis and HIV seroconversion. However, improved donor screening tests and newer virus attenuation and purification methods have resulted in much safer plasma-derived Factor VIII and Factor IX concentrates. Recombinant Factors VIII and IX concentrates are also available. Although the currently available plasma-derived Factor VIII concentrates seem to be safe in terms of both hepatitis and HIV, one cannot be absolutely sure of the safety of these products because of the risk of emerging infections. The majority of Factor VIII and Factor IX used in the United States is now recombinant (DNA-derived).

Desmopressin is considered the treatment of choice for bleeding in patients with vWD Type 1, with plasma-derived Factor VIII concentrates rich in the HMW multimers of vWF being used in those with Type 2 variants and Type 3 vWD. FFP (either solvent/detergent-treated or donor-retested) and cryoprecipitates are still used to treat many of the rare coagulation factor deficiencies, but aPCCs and rFVIIa are used more and more in rare bleeding disorders with good efficacy and limited side effects.

Venous and arterial thromboembolism in infancy and childhood have been described with increasing frequency over the past decade. The thrombus location, quality, and characteristics

of the patients (including age, genetic thrombophilia, immobility, etc) all affect the type and duration of treatment. LMW heparin or enoxaparin has been used to treat many pediatric patients with thromboembolism with good efficacy and minimal toxicities. The optimal type and duration of treatment for pediatric thromboembolism remains to be determined using prospective, randomized, controlled studies.

## Disclaimer

The authors have disclosed no conflicts of interest.

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# Management of Congenital Hemolytic Anemias

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The congenital hemolytic anemias comprise a heterogeneous group of intrinsic red cell abnormalities that may be conveniently classified as disorders of hemoglobin (hemoglobinopathies and thalassemia syndromes), red cell enzyme-deficiency disorders, and abnormalities of the red cell membrane and cytoskeleton. In patients affected with any of these conditions, Red Blood Cell (RBC) transfusions may be indicated to compensate for the decreased oxygen-carrying capacity associated with the underlying anemia. In addition, pathophysiologic consequences unique to each of these disorders may lead to other indications for transfusion. The hemoglobin-related abnormalities have special significance in clinical transfusion medicine, and particular emphasis is devoted to this group of disorders.

## Hemoglobinopathies and Thalassemias

The hemoglobinopathies are characterized by structural changes affecting the protein globin portion of the hemoglobin molecule, resulting from DNA mutations in the corresponding globin genes. Most forms of thalassemia are characterized by the production of diminished quantities of globin chains that are structurally normal. In certain globin-gene disorders, the molecular defect produces both a quantitative and a qualitative abnormality; examples include hemoglobin (Hb) E (a  $\beta$ -globin abnormality) and Hb Constant Spring (an  $\alpha$ -globin abnormality). This discussion on hemoglobin disorders focuses on sickle cell disease (SCD) and the  $\alpha$ - and  $\beta$ -thalassemias.

### Sickle Cell Disease

#### Epidemiology

The geographic distribution of the sickle globin ( $\beta^S$ ) gene in the Old World is shown in Fig 29-1. The greatest concentration of

individuals carrying this gene is localized in central West Africa, where the gene frequency for sickle hemoglobin (HbS) may be as high as 0.14. Appreciable numbers of affected individuals have also been identified in other parts of equatorial Africa, the Mediterranean, the Saudi Arabian peninsula, and India. This distribution substantially coincides with regions that historically have had endemic *Plasmodium falciparum* malaria. Ample epidemiologic evidence suggests that the  $\beta^S$  gene was subject to positive selection pressure because of the survival advantage that it confers in heterozygous individuals who are infected by the malarial parasite. In the New World, the  $\beta^S$  gene arrived as a result of the importation of slaves from Africa; in North America its gene frequency is approximately 0.04 to 0.05 among those of African ancestry. (Thus, approximately 10% of African Americans are sickle heterozygotes.)

#### Molecular and Cellular Pathophysiology

The fundamental defect in SCD is the substitution of thymine for adenine in the sixth codon of the gene for the  $\beta$ -globin chain, leading to a replacement of glutamic acid by valine at this site. In contrast to normal hemoglobin tetramers, HbS has an altered surface charge that promotes the formation of lengthy polymeric chains (gelation) when in the deoxygenated state. The oxygen affinity of dilute, unpolymerized HbS is similar to that of normal hemoglobin. However, the oxygen affinity of concentrated HbS solutions is decreased,<sup>2</sup> thereby representing a further stimulus for molecular polymerization.

Once a critical nucleation step has been achieved, the polymerization process may quickly progress to form rapidly lengthening fibers, which can further organize into filaments and even thicker strands. At the cellular level, this is reflected by the conversion of normal, deformable, biconcave red cells to rigid, highly viscous cells with the characteristic sickle shape. For the most part, the process of HbS gelation and erythrocyte sickling is a reversible one, but after repeated cycles these cells may become irreversibly sickled cells (ISCs). These ISCs maintain an abnormal sickled shape even in the absence of HbS polymerization and are probably a consequence of cumulative red

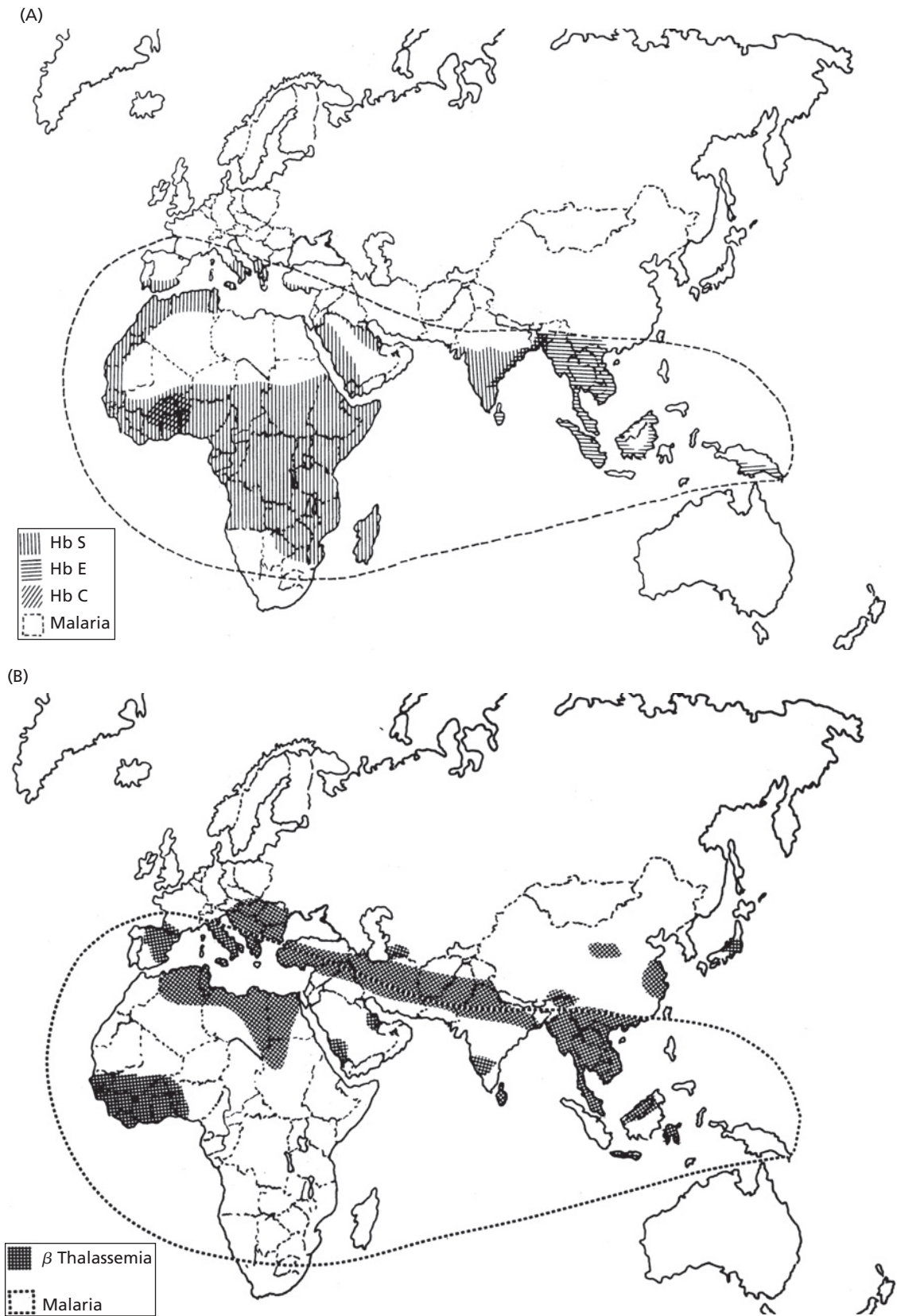


**Figure 29-1.** The geographic distribution of (A) hemoglobins S, C, and E and (B)  $\beta$ -thalassemia. The regions where *P. falciparum* malaria was formerly endemic are also indicated.<sup>1(p238)</sup>



cell membrane damage sustained during repeated sickle-unsickle cycles. Irreversibly sickled cells are much more rigid than biconcave red cells and have a much shorter life span, thus appreciably contributing to the hemolytic anemia that is characteristic of this disorder. Curiously, there is no close correlation between the percentage of circulating ISCs in an individual and the clinical severity of the disease.

As the process of sickling progresses and an increasing number of cells assume the sickle shape and become rigid, blood viscosity rises sharply. This change causes a marked delay in the transit of blood through the microvasculature, which in turn leads to increased oxygen extraction at the local tissue level. The resultant decrease in hemoglobin oxygen saturation further increases the propensity toward sickling, thus completing a self-propagating cycle.

A critical determinant for the initiation of the sickling process is the intraerythrocytic concentration of deoxygenated HbS (deoxy-HbS). As the concentration of deoxy-HbS rises, the delay time (ie, the time required to achieve a critical minimum polymer nucleus) diminishes exponentially, approximately on the order of the 15th to 30th magnitude.<sup>3</sup> Several genetic and cellular (erythrocyte) modulators of disease severity have been proposed and are reviewed below.<sup>4,5</sup> Some act by altering the intraerythrocytic concentration of HbS, whereas others exert their effect through unrelated and, in some cases, undetermined mechanisms.

Genetic factors that contribute most importantly to the severity of SCD include the concurrence of  $\alpha$ -thalassemia,  $\beta^0$ - or  $\beta^+$ -thalassemia, or any of several other hemoglobin abnormalities including HbC, HbD, HbO, and syndromes of hereditary persistence of fetal hemoglobin (HPFH) (see Sickle Cell Syndromes).

Other genetic determinants of the clinical expression of SCD appear to be related to the DNA “background” on which the sickle mutation is found. The  $\beta$ -globin gene cluster is located on the short arm of chromosome 11 and contains the  $\beta$ -,  $\delta$ -, and  $\gamma$ -globin genes, whose globin chain products, in combination with  $\alpha$  chains, form hemoglobins A, A<sub>2</sub>, and F, respectively. The DNA “haplotype” of a given region is defined by the local array of restriction endonuclease fragment-length polymorphisms. Three major DNA haplotypes have been described for the African  $\beta^S$ -globin region,<sup>6</sup> and it appears that at least certain measures of SCD severity may correlate with the different DNA haplotypes, probably in large part because of the differences they mediate in fetal hemoglobin (HbF) expression. Despite the influence that these genetic factors may have on the expression of SCD, they clearly are not the sole determinants of disease severity. Individuals with apparently identical globin genotypes can vary markedly in their clinical courses.

Several erythrocyte-related factors have also been implicated as possible modulators of disease severity.<sup>4,5</sup> They include the erythrocyte levels of 2,3-diphosphoglycerate (2,3-DPG; also known as 2,3-bisphosphoglycerate) or adenosine triphosphate, glucose-6-phosphate dehydrogenase (G6PD) deficiency, calcium or zinc deficiency, the ISC count, the degree of intraerythrocytic polymerization of deoxy-HbS, and the degree of cellular dehydration.<sup>4,5</sup>

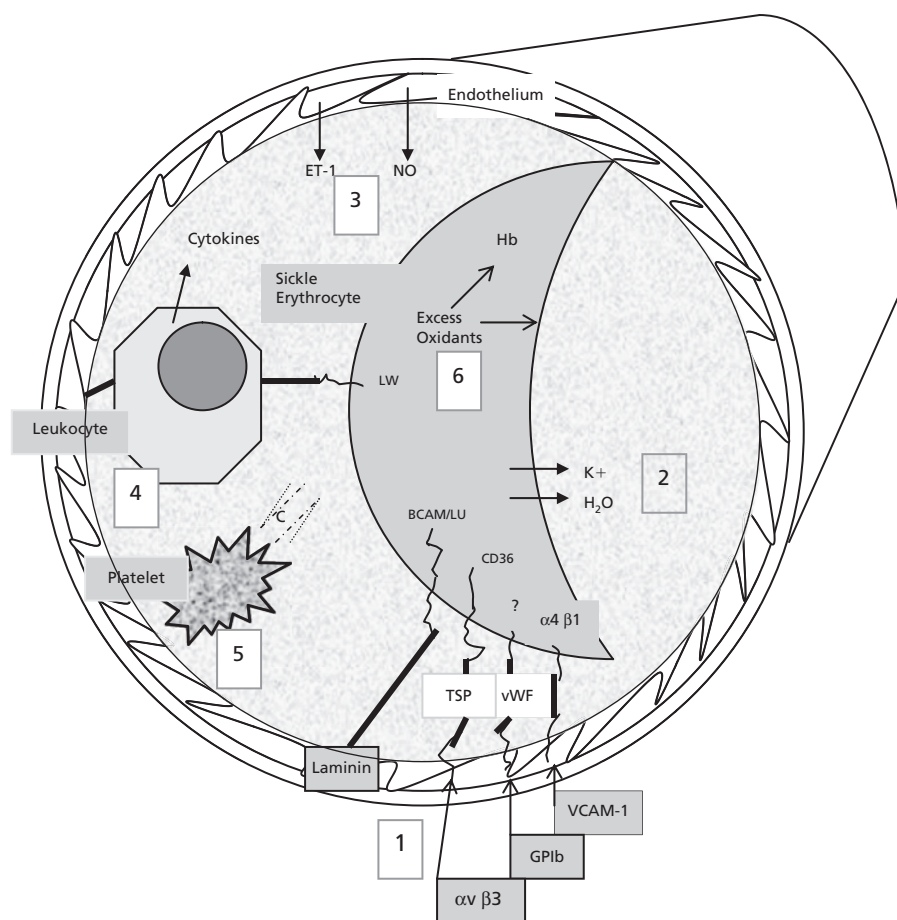
Increasingly, the role of extraerythrocytic factors in the vasoocclusive process is being appreciated. Sick erythrocytes have been shown to have abnormal interactions with coagulation and other plasma proteins, neutrophils, platelets, and vascular endothelium.<sup>7,8</sup> The consequence is a red cell environment characterized by abnormally adherent sickle erythrocytes, vascular inflammation, oxidant damage, and excessive stimulation of the coagulation system. Chronic intravascular hemolysis further contributes to these pathologic processes.<sup>9</sup> These abnormalities may lead to increased transit time through the microcirculation, or cause altered vascular tone; either consequence has the potential to enhance the likelihood of vasoocclusion. Certain interactions, such as those between neutrophils and the endothelium, result in inflammatory or oxidative injury to the vessel wall. Subsequently, damaged vascular endothelium contributes to increased adhesion of sickle erythrocytes. Thus, the vasoocclusive process is propagated by a cycle of events: sickle cell adhesion damages endothelium and vessel walls, and the damaged microvasculature in turn becomes a more potent nidus for adhesion, and ultimately, vasoocclusion. The interplay between sickle red cells and other factors in their environment that contribute to sickle vasoocclusion is illustrated in Fig 29-2.

Sickle red cells have increased adherence to vascular endothelium,<sup>11</sup> and this property appears to have a strong correlation with the severity of vasoocclusive disease.<sup>12</sup> Red cell adhesion occurs through direct interaction between erythrocyte and endothelial proteins, or through plasma proteins such as thrombospondin and von Willebrand factor, which act as bridges between red cell and endothelial binding sites. Elevated levels of red cell and endothelial binding sites, and plasma proteins contributing to adhesion, have been demonstrated in SCD.

Nitric oxide (NO) is an important vasodilator that is synthesized by the vascular endothelium,<sup>13</sup> and low levels in SCD contribute to the pathophysiology of complications such as acute chest syndrome (ACS) and stroke.<sup>14,15</sup> In addition, levels of the vasoconstrictor endothelin (ET-1) are elevated in SCD, and further contribute to increased vascular tone. Increased plasma homocysteine levels, probably related to folic acid deficiency, appear to be associated with an increased risk of stroke.<sup>16</sup>

### Mechanisms of Anemia

The anemia of sickle cell (HbSS) disease is characterized by increased peripheral destruction of the HbS-containing red cells. Progressive membrane damage to these spiny, brittle, poorly deformable erythrocytes is further intensified by secondary changes, including alterations in membrane lipid composition and oxidant damage. Erythrocyte production is substantially increased, in partial compensation for the rapid peripheral destruction. However, compensation is incomplete because of the rightward shift of the oxygen-hemoglobin binding curve that results from deoxy-HbS polymerization. The red cell life span in patients with SCD is approximately 5 to 20 days. Sickle cell complications, including acute splenic sequestration crisis (ASSC) and aplastic crisis, intensify the anemia (see Clinical Features).



Representative examples in each area are provided. Thick lines indicate binding. Please see the text for additional details.

1. Adhesion. Binding between membrane proteins on erythrocytes and endothelial cells is responsible for increased adhesion between the two. Circulating plasma factors, eg, thrombospondin (TSP) or von Willebrand factor (vWF), serve as bridges between these membrane proteins. Alternatively, direct binding may occur, eg, between  $\alpha 4 \beta 1$  integrin of the erythrocyte and VCAM-1 of the endothelial cell. In addition, erythrocytes may bind to laminin, an endothelial extracellular matrix protein. (VCAM-1 = vascular cell adhesion molecule 1; GPIb = glycoprotein Ib; BCAM/LU = basal cell adhesion molecule/Lutheran blood group; LW = LW blood group protein)
2. Erythrocyte membrane pump dysfunction. Loss of  $K^+$  and water result in intraerythrocytic dehydration, increasing the propensity for sickle polymerization.
3. Vasoconstriction/Vasodilation. The endothelium elaborates endothelin-1 (ET-1) and nitric oxide (NO). ET-1 is a potent vasoconstrictor, and may be produced in excess in sickle cell disease (SCD). NO is a potent vasodilator, and diminished production in SCD can lead to abnormal vasoconstriction. On the other hand, NO is normally inactivated by oxyhemoglobin. In a severe anemic state such as SCD, decreased hemoglobin concentration causes diminished inactivation, or relative excess, of NO. This may produce inappropriate localized vasodilation, and contribute to ventilation/perfusion mismatching seen in acute chest syndrome. NO may also inhibit endothelial adhesion of erythrocytes and platelet aggregation.
4. Leukocytes. Leukocytes in SCD are often increased in number, and demonstrate increased adhesion to both endothelium and erythrocytes. They are a source of cytokines and other mediators of inflammation.
5. Platelets. Increased platelet adhesion and aggregation, and a thrombogenic state of coagulation factors (C) may impede flow.
6. Oxidant damage. Increased generation of oxidants by sickle erythrocytes leads to denaturation of hemoglobin (Hb) and membrane lipid peroxidation.

**Figure 29-2.** Schematic representation of factors potentially influencing the vasoocclusive process.<sup>10</sup>

In addition, other conditions common in sickle cell patients contribute to anemia, including fever or infection or both, immune hemolysis, G6PD deficiency, and folic acid deficiency.

### Sickle Cell Syndromes

Because of the prevalence of other  $\alpha$ - and  $\beta$ -globin mutations within the African sickle cell population, SCD is a heterogeneous

group of disorders. In each individual syndrome, the primary determinants of sickling are the intracellular concentration of HbS and the propensity of other nonsickle globins present within the cell to participate in the polymerization process. Fetal hemoglobin is virtually totally resistant to incorporation into sickle globin polymers, whereas both adult hemoglobin (HbA) and HbC participate to some degree in this process.

**Table 29-1.** Hematologic Values Associated with Common Sickle Cell Syndromes in Adults

| Hemoglobin Genotype        | Hemoglobin Concentration (g/dL) | Mean Cell Volume (fL) | HbF (%) |
|----------------------------|---------------------------------|-----------------------|---------|
| HbAA (normal)*             | 12-18                           | 80-100                | 1       |
| HbAS*                      | 12-16                           | 82-92                 | 1       |
| HbSS                       | 6.5-9.5                         | 80-98                 | 2-20    |
| HbS/ $\beta^0$ thalassemia | 6.0-12.5                        | 63-88                 | 1.4-20  |
| HbS/ $\beta^+$ thalassemia | 6.5-14.0                        | 62-84                 | 1-15    |
| HbSC                       | 8.5-12                          | 82-92                 | 1-8     |
| HbS/HPFH†                  | 7-18                            | 75-89                 | 15-85   |
| HbSS/ $\alpha$ -thal trait | 7.5-10                          | 60-80                 | 5-20    |

\*No associated clinical disease; included for comparison.

†Hereditary persistence of fetal hemoglobin.

However, patients with HbSC disease have a higher percentage of HbS in their erythrocytes than do individuals with sickle cell trait (HbAS). In addition, HbSC erythrocytes have a membrane defect that leads to intracellular dehydration, causing a further increase in the intraerythrocytic hemoglobin concentration.<sup>17</sup> Because of both these factors, HbSC erythrocytes are far more likely to undergo sickling than are HbAS cells. As would be predicted from these differences, the relative expected disease severity is: HbSS, HbS/ $\beta^0$  thalassemia > HbSC, HbS/ $\beta^+$  thalassemia > HbS/HPFH > HbAS. Concomitant  $\alpha$ -thalassemia in individuals with HbSS disease does not appear to impose a significant change in the frequency of pain crises. However, some forms of chronic organ damage are increased in this disorder because of higher hemoglobin levels, which could lead to increased blood viscosity.<sup>18</sup> Characteristic hematologic indices of some of the important sickle syndromes are shown in Table 29-1.

### Transfusion Therapy in Sickle Cell Disease

The chronic anemia of SCD is usually well tolerated, but during intercurrent illnesses RBC transfusions may be required. Transfusions may also be indicated in order to limit or prevent vasoocclusive complications, either acute or chronic. As indicated in Table 29-2, some of the complications of SCD are characterized by both anemia and vasoocclusive manifestations. In general, for complications in which anemia is the predominant concern, traditional transfusion goals such as adequate hemoglobin concentration are appropriate. In contrast, for complications in which sickle cell vasoocclusion is the overriding concern, the primary objective of RBC transfusion is to achieve a favorable balance between the relative concentrations of normal and sickle red cells.

### Transfusion in Patients with Sickle Cell Vasoocclusion

The purpose of transfusion in the management of sickle cell vasoocclusion is to diminish the likelihood of intravascular sickling through the dilution and replacement of the recipient's sickle cells by transfused, nonsicklable cells. Clinical experience has shown that vasoocclusion is unlikely to occur with mixtures of AA (normal) and SS (sickle) cells if the relative concentration

**Table 29-2.** Acute and Chronic Complications of Sickle Cell Disease that May Require Transfusion Because of Anemia or Vasoocclusion

|         | Anemia                                                             | Anemia and Vasoocclusion                                                                                                                  | Vasoocclusion                                                                                                          |
|---------|--------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------|
| Acute   | Aplastic crisis                                                    | Acute chest syndrome<br>Acute splenic sequestration<br>Acute papillary necrosis<br>Acute multiorgan failure syndrome<br>Sepsis<br>Surgery | Stroke<br>Priapism<br>Intractable pain crisis                                                                          |
| Chronic | Pregnancy<br>Hematuria<br>Chronic renal failure<br>Cardiac failure | Pregnancy, high risk<br>Pulmonary artery hypertension                                                                                     | Skin ulcer<br>Prophylaxis for:<br>Stroke (primary, secondary prophylaxis)<br>Splenic sequestration<br>Intractable pain |

of HbS-containing cells is less than 30% to 40%. This finding is supported by in-vitro data from Lessin et al,<sup>19</sup> who showed that the relative resistance to flow of mixtures of AA and SS red cells rose sharply when the proportion of SS cells exceeded 40%. Schmalzer et al<sup>20</sup> showed that at a fixed concentration of sickle cells ( $H_S/H_T$  or  $H_S$  alone, where  $H_T$  is the total hematocrit and  $H_S$  is the hematocrit of sickle cells), the viscosity ( $\eta$ ) rose with the hematocrit ( $H_T$ ), because of the addition of AA cells (Figs 29-3 and 29-4). These authors also showed that deoxygenation does not change the viscosity of AA cells but causes appreciable increases in the viscosity of SS cells, especially at higher hematocrits (Fig 29-3). The rise in viscosity was steepest with higher  $H_S$  values, and at fixed absolute ( $H_S$ ) or relative ( $H_S/H_T$ ) concentrations of sickle cells the effective oxygen delivery ( $H_T/\eta$ )<sup>20</sup> declined when  $H_T$  rose through the physiologic range (20% to 40%) (Fig 29-5). This effect, too, was more pronounced with higher  $H_S$  (or  $H_S/H_T$ ). These results indicate that in suspensions of normal cells ( $H_S$  or  $H_S/H_T = 0$ ), as  $H_T$  rises, the benefit provided by increased oxygen-carrying capacity is nearly equally

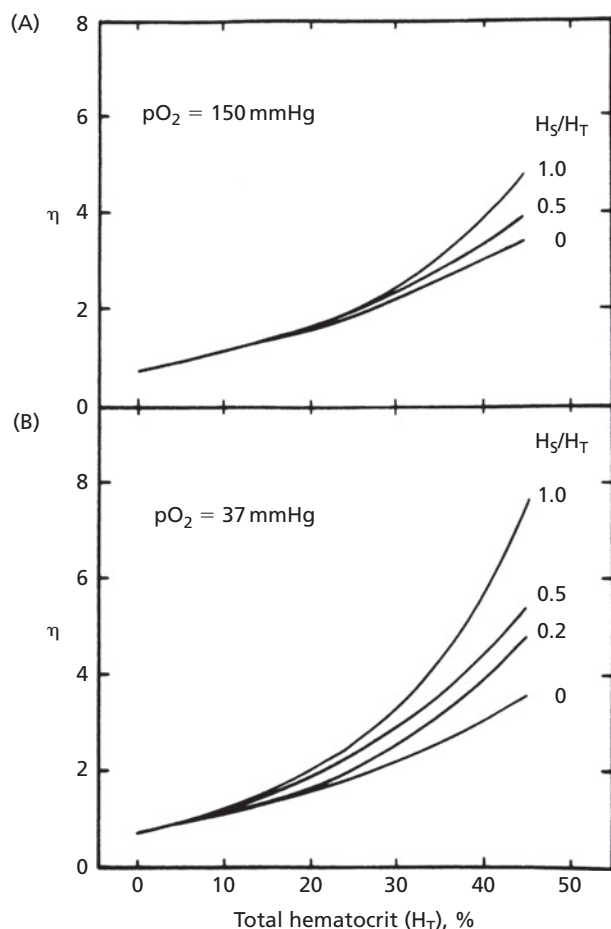


**Figure 29-3.** The rise of viscosity ( $\eta$ ) with  $H_T$  at given proportions of sickle cells to total cells ( $H_S/H_T$ ) in the suspension. (See text for definition of terms.)  
(A) Oxygenated cell suspensions. (B) Deoxygenated suspensions.<sup>20</sup>

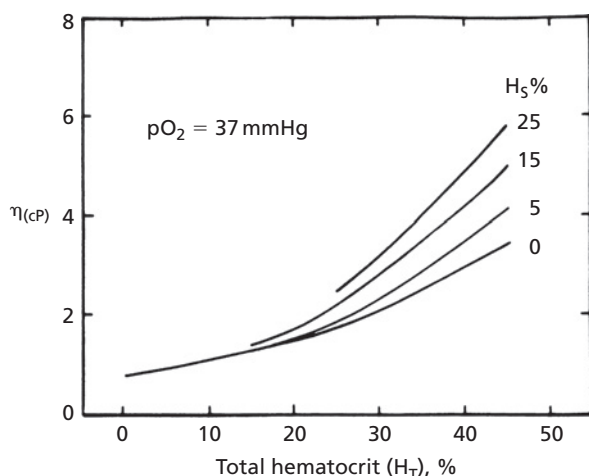


**Figure 29-4.** The data from Fig 29-3 (B) replotted to show the rise in viscosity ( $\eta$ ) with  $H_T$  at various hematocrit levels of sickle cells ( $H_S$ ).<sup>20</sup>

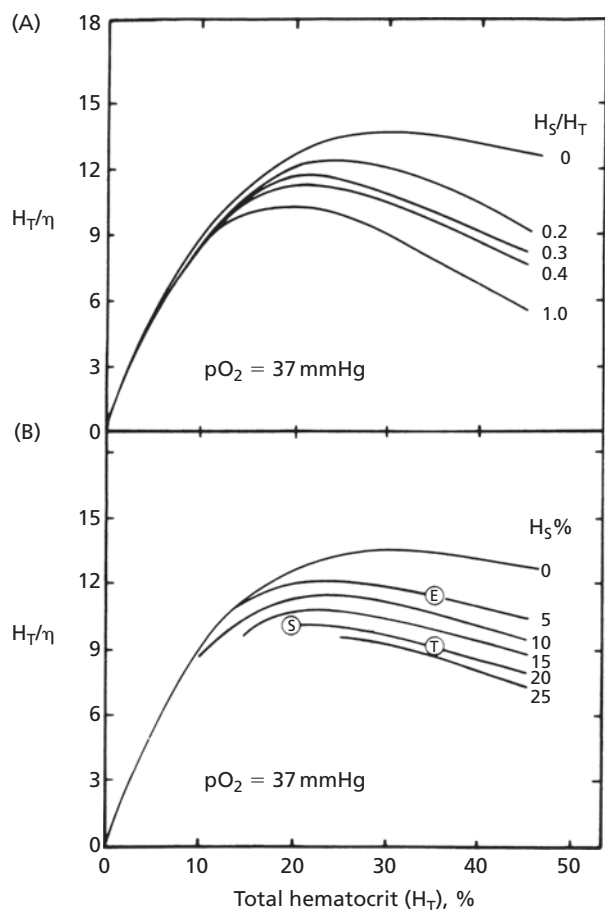


**Figure 29-5.** The  $H_T/\eta$  ratio for suspensions containing mixtures of AA and SS cells. (A) Increasing  $H_S/H_T$  ratios cause a lowering of the  $H_T/\eta$  curve. (B) The same data as in A is plotted with constant levels of  $H_S$ . The relative merits of simple vs exchange transfusion can be analyzed by using the curves for  $H_T/\eta$  (B) to illustrate a hypothetical patient. *Point S* represents a patient with sickle cell disease with  $H_T = H_S = 20\%$  before transfusion treatment. After simple transfusion to raise the  $H_T$  to  $35\%$ , *Point S* moves along the same  $H_T/\eta$  curve ( $H_S = 20\%$ ) to *Point T*, and there is a drop of about 11% in oxygen delivery ( $H_T/\eta$ ). However, if an exchange transfusion is carried out to lower the  $H_S$  level of the patient to 5%, although the  $H_T$  is raised to  $35\%$  with AA cells, the result would be at *Point E*, where the  $H_T/\eta$  is 11% more than at *Point S* and about 23% more than at *Point T*.<sup>20</sup>

offset by the adverse effect of increased viscosity, so that the effective oxygen delivery remains nearly constant (Fig 29-5). Conversely, in the presence of sickle cells (higher  $H_S$  or  $H_S/H_T$ ), the effect upon viscosity predominates and a net decrease in the effective oxygen delivery results. These data strongly emphasize the rheologic advantages of exchange transfusion over simple transfusion when used in the management of patients with sickle cell vasoocclusion. Either type of transfusion produces relative enrichment of normal over sickle cells, but the exchange method has the added benefits of rapidly diminishing  $H_S$  and limiting the rise in  $H_T$ . Both effects are helpful in reducing viscosity and improving oxygen delivery to tissues (Fig 29-5).

The distribution of HbS among the erythrocytes significantly influences the overall likelihood of sickling. For example, in mixtures of AA and SS erythrocytes, the SS cells fully retain the capacity to sickle, whereas AA cells, of course, lack any potential to sickle. Similarly, in HbSC disease or other clinically significant compound heterozygous condition, each HbS-containing cell retains the potential to sickle under suitable physiologic conditions. When planning transfusions for managing sickle vaso-occlusion in individuals with compound heterozygous conditions, it is therefore most prudent not to focus on the percentage of the HbS, but rather to consider the percentage of cells that are sicklable. For example, if the recommendation for a particular indication in HbSS disease is to achieve a HbS concentration of 30%, it should be reinterpreted in HbSC disease to mean that transfusion should achieve 30% sicklable cells (yielding approximately 15% HbS) or an HbA concentration of 70%.

### Clinical Features

At birth, infants with homozygous (HbSS) SCD are clinically and hematologically normal because of the predominance of HbF during this period. During the ensuing several months, as the percentage of HbF naturally declines, anemia and other hematologic abnormalities become increasingly apparent. Susceptibility to clinical sequelae of SCD typically begins at 3 to 6 months of age. Various clinical findings in young children with SCD, including early onset of dactylitis (“hand-foot syndrome”), severity of anemia, and presence of leukocytosis, may be predictive for disease severity and mortality,<sup>21,22</sup> but some of these relationships (eg, early onset dactylitis) have not been found consistently.<sup>23</sup>

### Acute Sick Cell Crises

The hallmark clinical expressions of SCD are acute sickle cell “crises.”

**Acute Pain Crisis.** These episodes, which are believed to result from vasoocclusion, account for the majority of hospitalizations of sickle cell patients in the United States. Pain crises may involve practically any area of the body but most often are musculoskeletal or soft tissue in origin. These episodes may be brought on by a variety of initiating conditions, including fever, infection, acidosis, and hypoxia, but frequently there is no identifiable precipitant. Occasionally, pain crises are so severe as to be unresponsive to high-dose narcotic analgesia, and they often occur with great enough frequency to be debilitating and severely disruptive of school or work. Acute dactylitis involves the distal extremities and characteristically occurs in infancy. Frequently, this complication represents the initial identifiable clinical event in children with SCD.

RBC transfusions are rarely indicated for the treatment of severe or protracted episodes of acute pain crisis or for the prevention of frequent recurrences. When severe acute pain crisis is unresponsive to the standard therapy of intravenous hydration and analgesia, exchange transfusion designed to lower the HbS to less than 40% to 50% may produce relief. Debilitating cycles of frequent pain crises may be arrested by regular courses of

transfusion that maintain the HbS at less than 40% to 50%. However, this approach is associated with a high risk of recurrence when the transfusions are stopped.

**Acute Splenic Sequestration Crisis.** In patients with SCD, repetitive microocclusive events occur in the spleen throughout early childhood, so that by adulthood the spleen is often “autoinfarcted” and lacks any appreciable blood circulation. Before reaching this state, and primarily in early childhood, the spleen is subject to acute obstruction by sickle cells in the efferent circulation. In about one-half of reported cases, there is a history of infectious illness. The period of risk for acute splenic sequestration is typically prolonged until the early adult years in patients with the milder sickle syndromes (eg, HbSC and HbS/ $\beta^+$ -thalassemia).

Splenic sequestration is a rapidly progressive and potentially fatal process. The clinical course may progress from a state of relative well-being to circulatory shock within just a few hours. Upon presentation, the hemoglobin concentration may be as low as 2 g/dL. Volume resuscitation can be initiated with crystalloid solution followed by prompt transfusion of RBC units. The volume of RBC transfusions should be sufficient to restore baseline hemoglobin levels. One or 2 days following transfusion, splenomegaly will typically diminish and the hemoglobin will spontaneously rise, indicating resolution and liberation of formerly sequestered blood cells into the circulation.

In contrast to the typical, rapidly progressive course of acute splenic sequestration crisis (ASSC), subacute, minor episodes may be seen, especially in children with chronic hypersplenism. If splenectomy is being considered to avert recurrence of ASSC or hypersplenism, a limited transfusion program of 1 year may resolve the disease process and avoid surgery. At the least, transfusion can stave off surgery until the patient reaches an age when splenectomy would pose less risk of sepsis. As in other long-term transfusion programs intended to prevent sickle vasoocclusion, the HbS concentration should be maintained at less than 30% to 40%. However, repeated ASSC episodes have even been reported with HbS concentrations as low as 14% to 16%.<sup>24</sup>

**Aplastic Crisis.** This complication typically occurs in children of an age and history similar to those of children who experience splenic sequestration. Aplastic crisis is a transient, self-limited cessation of erythrocyte production caused in most, if not all, cases by infection with human parvovirus.<sup>25</sup> Because the life span of sickle red cells is only 5 to 20 days, any temporary cessation of erythrocyte production can lead to severe anemia within a short time.

Aplastic crisis is characteristically self-limited, and spontaneous recovery usually occurs within a few days of onset. Decisions regarding transfusion must take into account the patient’s clinical status as well as the hemoglobin concentration and any evidence of recovery, as reflected by the reticulocyte count. When possible, an early decision about transfusion helps to diminish the period of mandatory close observation and shorten the hospital stay. Transfusion to or near the baseline hemoglobin level is sufficient, and under such circumstances there need not be concern that transfusion will delay the recovery from marrow aplasia.

### Stroke

In addition to the pain episodes that apparently result from vasoocclusion, patients with SCD may also experience major, in some cases catastrophic, ischemic events. Sick cell vasoocclusion in the cerebral circulation may cause cerebrovascular accidents, producing hemiplegia, seizures, coma, or death. In children, strokes usually are caused by cerebral infarction, whereas subarachnoid or cerebral hemorrhages become more prevalent with advancing age. Over two-thirds of strokes in patients with SCD occur during childhood, and approximately 11% of children with SCD suffer from stroke.<sup>26</sup> In the absence of further intervention, approximately two-thirds of sickle cell patients with stroke will have a recurrent episode, usually within 3 years.<sup>26</sup> Functional recovery after a first stroke is variable, and subsequent episodes may produce additional and frequently more devastating sequelae. The acute mortality in adult patients is especially high and may approach 50%. Acute cerebral events that produce focal neurologic deficits lasting less than 24 to 48 hours—transient ischemic attacks (TIAs)—may be a harbinger of stroke in these patients. In sickle cell patients, chronic neurologic damage, manifest as silent cerebral infarcts (SCIs) on magnetic resonance imaging, is twice as common as overt stroke, and is associated with impaired cognitive function.<sup>27</sup> SCI is a risk factor for the development of stroke.

The primary acute therapy for stroke in patients with SCD is exchange transfusion to limit further intracerebral sickling. Exchange transfusion is preferred rather than simple transfusion because it allows a greater reduction in the fraction of circulating sickle cells, and it more rapidly improves the rheologic properties of circulating blood. In addition, the risk of stroke recurrence appears to be lower with exchange rather than simple transfusion.<sup>28</sup> At a minimum, a single-volume exchange should be performed, with the aim of achieving a HbS concentration of approximately 30%. In more severe cases, a greater level of protection may be desirable during the acute phase; a double-volume exchange would be expected to reduce the level of HbS to approximately 10%.

The recurrence rate of stroke in sickle cell patients is high. Chronic transfusion to maintain the HbS concentration below 30% to 40% has been effective in reducing the incidence and severity of recurrent neurologic events.<sup>29</sup> However, this beneficial effect may be temporary, lasting only for the duration of transfusion therapy. For example, the incidence of recurrence is reduced to approximately 10% during transfusion therapy but increases again within 1 year after the cessation of transfusions.<sup>30</sup> When transfusions are stopped after 5 to 12 years, the risk of stroke recurrence still remains elevated.<sup>31</sup> The appropriate duration of therapy, therefore, remains a vexing clinical question. Common practice is to continue transfusion until 18 years of age, if not longer.<sup>32</sup>

The following management of stroke is advised. Acute exchange transfusion aims to achieve rapid reduction of circulating sickle cells to less than 30%. If cerebral angiography is planned, a further decrease of HbS to 20% should be achieved.

Chronic transfusion therapy to maintain the HbS concentration at less than 30% is advised for at least 5 years. In order to diminish the transfusion load, it may be possible to relax the transfusion criteria after 5 years and maintain the level of HbS at less than 50%.<sup>33</sup> Beyond that period, as noted above, transfusions should be continued at least until the patient is approximately 18 years of age, and possibly indefinitely. Transfusion therapy should be monitored by hemoglobin fractionation for determination of the percentage of HbA and HbS, as well as hemoglobin and reticulocyte determinations. If frequent fractionation measurements are impractical, it may be possible to estimate an individual's transfusion requirement with reasonable accuracy by measuring the reticulocyte count and hemoglobin level after the patient's individual pattern has been established.<sup>34</sup> Complications resulting from transfusions, such as hepatitis, alloimmunization, and iron overload may necessitate early cessation of chronic transfusion. Improvements in cerebral radiographic findings should not be misinterpreted as evidence that risk of stroke recurrence has abated.

All these treatment considerations unfortunately underscore a great deficiency in the attempt to prevent stroke in sickle cell patients—they are designed to prevent a second stroke, but ignore the considerable morbidity that occurred as a result of the first event. Is there a reliable means to identify prospectively those patients who are at high risk for developing a first stroke, so that they can be offered prophylactic therapy? Children with elevated cerebral blood flow velocity, as measured by transcranial Doppler (TCD) ultrasonography, have an increased risk for developing stroke during the several years following abnormal studies. On the basis of this screening method, the Stroke Prevention Trial in Sickle Cell Anemia ("STOP") tested the hypothesis that regular prophylactic transfusions in patients with abnormal TCD findings could prevent a first stroke.<sup>35</sup> A total of 1934 children ages 2 to 16 years were screened using TCD, and 206 were found to have abnormal results. Of 130 children who were deemed suitable for the study, 63 patients were randomly assigned to receive regular transfusions designed to maintain the concentration of HbS at less than 30%, and 67 patients were assigned to receive standard care (no transfusions). During the course of the trial, 10 patients dropped out of the transfusion group, and two patients assigned to standard care transferred to the transfusion group. After a follow-up period of up to 30 months, there were 10 cerebral infarctions and one intracerebral hematoma in the standard care group, compared with one infarction in the transfusion group. Because of the obvious benefit afforded by prophylactic transfusion, this study was terminated early in order to allow patients in the standard care group to elect to receive regular transfusions.

Patients who received such primary stroke prophylaxis were more likely to revert to normal TCD findings,<sup>36</sup> suggesting that chronic transfusion can actually aid in the healing of abnormal vasculature. Is it safe to stop prophylactic transfusion after TCD velocities have normalized? A follow-up study ("STOP 2") addressed that question, and found that once preventive transfusions were stopped, many patients again developed abnormal TCD

results, or even stroke, within just a few months.<sup>37</sup> A recommended duration for primary stroke prevention has not been established. In patients with abnormal TCD results and evidence of SCI, chronic transfusion has also been demonstrated to lower the risk of new SCI.<sup>38</sup> Whether transfusion of patients with a history of SCI but normal TCD results could lower the incidence of new SCI is currently under study (“Silent Infarct Transfusion or SIT Trial”).<sup>39</sup>

Despite these promising results, many questions remain unanswered. That regular transfusions are highly effective in preventing stroke is hardly surprising. The crucial question is, given the limitations of TCD methodology, do the benefits of primary stroke prevention via chronic transfusion outweigh the risks? The positive predictive value of high-risk TCD results is only about 35% to 40%,<sup>40,41</sup> implying that a majority of patients deemed as high risk for stroke by TCD testing would be subjected to the risks of chronic transfusion unnecessarily. A recent analysis employed computer modeling to hypothetically compare the projected benefits and risks of several common primary stroke prevention strategies.<sup>42</sup> The key parameter assessed in this study was life expectancy. Several assumptions were employed in this study, including an 80% participation rate of families for whom preventive transfusions were recommended, use of the oral medication deferasirox for iron chelation, and an 80% compliance rate with that treatment. This study found that all the commonly recommended primary stroke prevention strategies employing transfusion resulted in a shorter life expectancy than no transfusion at all. In all cases, but to varying extents, the life expectancy gain produced by averting strokes was outweighed by increased mortality from iron overload. The decision model highlighted the major adverse impact that iron accumulation has in any long-term transfusion regimen. Indeed, in the analysis, if 100% compliance with oral iron chelation was assumed instead of 80%, then the adverse impact of iron accumulation was averted in virtually all the analyzed regimens. Some limitations of this study should be recognized. Some of the assumed benefits of primary stroke prevention may have been overstated because the data employed were derived from controlled clinical trials, in which rates of adherence, monitoring, and follow-up are undoubtedly better than those that would be achieved in general clinical settings. Conversely, some benefits of chronic transfusion may have been understated, because this study focused on life expectancy as an outcome, and did not address any potential improvements in quality of life.

Ultimately, it is perhaps understandable that primary stroke prevention with chronic transfusion has met considerable resistance from physicians and sickle cell families.<sup>41</sup> Alternative, effective medical strategies certainly would be highly welcome. Hydroxyurea has been found capable of lowering TCD flow velocities,<sup>43</sup> but it may not be sufficiently reliable to be clinically useful.<sup>44</sup>

### Acute Chest Syndrome

The clinical picture of ACS includes new pulmonary infiltrates on chest radiograph, often accompanied by a combination

of fever, cough, chest pain, and hypoxia. Infection is the most common cause in young children, whereas vasoocclusion is probably the most likely etiology in adults.<sup>45</sup> Especially in children, one complication can precipitate the other. Regardless of the underlying cause, the clinical course can be extremely variable; the most severe cases can progress to an acute respiratory distress syndrome (ARDS)-like picture within just a few hours.

Among those patients with an infectious etiology of ACS, the most common pathogens are *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, and respiratory syncytial virus. Pulmonary fat embolism, presumably caused by infarction of bone during a vasoocclusive event, is another important cause of ACS.<sup>46</sup> Serum levels of secretory phospholipase A<sub>2</sub> (sPLA<sub>2</sub>), an enzyme that hydrolyzes phospholipids into free fatty acids and lysophospholipids, has been described as a useful biochemical marker that is markedly elevated during episodes of ACS, and correlates with its clinical severity.<sup>47</sup> sPLA<sub>2</sub> levels rise 24 to 48 hours before clinical presentation of ACS, and thus it is a useful tool to predict impending disease<sup>48</sup>; serum levels of C-reactive protein, a much more readily available test, appear to parallel those of sPLA<sub>2</sub>.<sup>49</sup>

RBC transfusions are often necessary for the treatment of this complication if it is associated with hypoxemia. Prompt treatment helps to avert clinical deterioration. In critical situations, a single-volume exchange transfusion is recommended, because this approach poses no risk of volume overload and achieves a rapid replacement of sickle cells with normal red cells. In less urgent situations, simple transfusions are usually satisfactory.

### Hepatic Crisis

Sickling in the liver produces sharp right upper quadrant abdominal pain, hyperbilirubinemia, and other abnormal liver function values. The clinical picture often resembles that seen in acute cholelithiasis. Because asymptomatic gallstones are coincidentally seen in approximately one-half of all young adult patients with SCD, it is important to distinguish between the two conditions. Treatment with supportive care generally suffices.

Acute hepatic sequestration has been rarely described, and is characterized by a rapidly enlarging liver accompanied by a significant decrease in the hemoglobin concentration. RBC transfusion may be required; in order to prevent hyperviscosity stemming from release of sequestered cells, exchange transfusion is preferred.

### Priapism

Prolonged, painful, and unwanted penile erection occurs in up to 89% of male patients with SCD<sup>50</sup> and appears to be most prevalent in sickle cell patients who have unusually high rates of hemolysis.<sup>51</sup> Common clinical pictures include prolonged erection (lasting more than 4 hours), which often results in severe pain, difficulty in urination, and even impotence, or brief episodes lasting only a few hours, which may be only moderately painful with no long-term sequelae, and which may occur in clusters (“stuttering priapism”).

Two types of priapism have been described based on blood flow patterns in the penile vasculature. High-flow priapism is caused by dysregulated arterial inflow stemming from cavernosal arterial injury, and is uncommonly associated with ischemia or pain. High-flow priapism rarely requires urgent treatment. In contrast, low-flow priapism is thought to be caused by venous sickle vaso-occlusion within the corpora cavernosa, and is often associated with localized ischemia and pain. This type is much more common in SCD, and requires prompt attention to avert long-term sequelae.<sup>52</sup>

In milder priapism of very recent onset, intravenous hydration and analgesia are the mainstays of therapy, but this approach is often insufficient. If priapism has lasted longer than 4 hours, then aspiration of the corpora cavernosa under local anesthesia with instillation of epinephrine, a mixed  $\alpha$ - and  $\beta$ -agonist, has proven to be effective.<sup>53</sup> Instillation of phenylephrine, which primarily has  $\alpha$ -agonist activity, can be used instead of epinephrine, and might be preferable.<sup>52</sup>

Simple and exchange transfusion have been employed in the management of acute priapism, but their role is unclear. Seeler<sup>54</sup> obtained satisfactory results by transfusing sufficient RBC units to double the hemoglobin concentration. In more severe or prolonged episodes (those lasting longer than 6 to 12 hours), a single-volume exchange transfusion may be considered. If this is successful, relief of pain occurs relatively soon, but complete detumescence may take several days or even weeks. If this approach is unsuccessful, a surgical shunt between the corpora cavernosa and corpus spongiosum may relieve the circulatory obstruction. If surgery is required, an exchange transfusion will have prepared the patient for anesthesia and surgery (see Special Indications for Transfusion). However, acute neurologic events, including seizure and stroke, have been reported in children who have undergone partial exchange transfusion for priapism, and the association between the two has been termed the ASPEN syndrome (association of sickle cell disease, priapism, exchange transfusion, and neurologic events).<sup>55</sup> This complication frequently begins with acute onset of severe headache. The etiology of these neurologic complications is not well understood, but it has been speculated that release of vasoactive substances is involved.<sup>52</sup> If RBC transfusions are used in the management of acute priapism, it is important to ensure that they not delay the use of more definitive therapies, such as intracavernosal injection, or shunt placement.

### **Hematuria**

Significant urinary blood loss from acute papillary necrosis in patients with SCD may require transfusions for correction of anemia. It is unclear whether transfusion accelerates healing of the renal papillae in these patients. Acute papillary necrosis has also been described in patients with sickle cell trait.

### **Infection**

Patients with SCD have impaired host defense function and increased susceptibility to infections, particularly to *Streptococcus pneumoniae* and *Haemophilus influenzae b*. In addition, bouts of infection are considerably more severe and protracted in patients

with SCD, compared to normal individuals. Sepsis may hinder erythropoiesis, as well as accelerate hemolysis. In severe infections, the red cell T cryptantigen may be exposed, leading to red cell polyagglutination.<sup>56</sup> Transfusions may be indicated in sickle cell patients with sepsis in order to improve oxygen-carrying capacity and to prevent widespread sickling that might result from associated hypoxia or acidosis.

### **Acute Multiorgan Failure Syndrome**

This relatively uncommon but life-threatening complication typically occurs after an episode of unusually severe pain crisis, and is probably caused by widespread vasoocclusion. Acute failure develops in at least two of three organs: lungs, liver, or kidney. The onset of organ failure is often accompanied by fever, rapid decrease in hemoglobin concentration and platelet count, nonfocal encephalopathy, and rhabdomyolysis.<sup>57</sup> It is curious that this catastrophic complication seems to preferentially affect patients who previously had relatively mild disease, relatively high steady-state hemoglobin levels, and no prior chronic organ damage. Aggressive transfusion therapy, either through the use of repeated simple transfusions, and/or exchange transfusions, is associated with improved survival in this severe complication.<sup>57,58</sup>

### **Chronic Effects of Sickle Cell Disease**

Chronic organ damage from SCD produces an additional spectrum of disease manifestations. RBC transfusions play a role in the management of these complications.

**Cardiac Effects.** Cardiomegaly, especially left ventricular hypertrophy, most often reflects chronic anemia, but may also result from sickle cardiomyopathy. Even though the coronary circulation is a site of high oxygen extraction, the incidence of myocardial infarction is diminished in sickle cell patients. Apparently, the short transit time through the coronary circulation is less than the delay time for sickling.

**Pulmonary Effects.** Pulmonary arterial hypertension (PAH) affects about a third of adult patients with SCD, and is associated with a significantly increased risk of death.<sup>59</sup> PAH is diagnosed by Doppler echocardiography, with a finding of tricuspid regurgitant jet velocity of at least 2.5 m/second. This condition is strongly associated with increased rates of hemolysis, a finding which supports the pathophysiologic process alluded to previously (see Molecular and Cellular Pathophysiology). Hemolysis is injurious to endothelium, and contributes to increased vascular tone by lowering levels of NO.<sup>9</sup> Chronic lung disease with ventilation-perfusion mismatch or reduction in lung volume may result from repeated or severe episodes of pneumonia or pulmonary infarction.

Sickle cell patients with the most severe PAH are treated with a variety of medical therapies, including oral selective pulmonary artery vasodilators, prostacyclin analogues, hydroxyurea, and inhaled NO.<sup>60</sup> In addition, they are often treated with exchange transfusions.<sup>59</sup> Preliminary data indicate that early stages of PAH may be reversed by transfusion therapy.<sup>61</sup>



**Hepatobiliary Effects.** Cholelithiasis occurs in approximately 40% to 50% of patients with SCD by the time they reach young adulthood, but in the majority of cases it is asymptomatic. Cholecystectomy is required for those patients who are symptomatic, but many have managed asymptomatic patients expectantly; some however, do advocate elective cholecystectomy for those with cholelithiasis who are asymptomatic.<sup>62</sup>

**Renal Effects.** The renal medulla is a hypertonic, acidotic environment that readily encourages intravascular sickling. Beginning in early childhood, intrarenal ischemia leads to an impaired ability to concentrate urine maximally (hyposthenuria). Infrequently, patients with SCD also develop nephrotic syndrome and overt renal failure. Their anemia may also worsen in the face of frank renal failure.

**Skeletal Effects.** Avascular necrosis of the femoral head produces Legg-Perthes-like radiographic changes, and is a common cause of chronic pain and debilitation in patients with SCD. This complication is associated with higher hemoglobin levels and concomitant presence of  $\alpha$ -thalassemia. Sick cell-induced infarction of the vertebral bodies is a cause of severe back pain, and is seen as a “fishmouth” appearance of the vertebrae on radiographic examination.

**Dermal Effects.** Adolescent and adult sickle cell patients are prone to develop leg ulcers, usually in the anterior tibial region or adjacent to the medial malleolus. Infection is common, and healing occurs slowly; the recurrence rate is high. When severe, leg ulcers adversely affect mobility, employability, and overall quality of life.

When local measures are unsuccessful in the management of severe ulcers, a transfusion program lasting several months may achieve satisfactory results.<sup>63</sup> However, the role of transfusion in the management of skin ulcers is debatable.<sup>58</sup>

**Ophthalmic Effects.** Patients with SCD are at increased risk for ophthalmic disease, which occurs in numerous forms.<sup>64</sup> Nonproliferative lesions, which frequently can be identified by direct ophthalmoscopic examination, affect a variety of structures, including the retina, macula, choroid, and optic disc. Proliferative retinopathy occurs primarily in adolescent or older patients with HbSC or HbS/ $\beta^+$ -thalassemia, and indirect ophthalmoscopy and fluorescein angiography are used to detect the characteristic retinal vascular changes. In severe cases, there can be progression to vitreous hemorrhage and retinal detachment, and even blindness. However, spontaneous resolution of milder disease occurs quite commonly,<sup>65</sup> and so optimal management of proliferative sickle retinopathy is not always clear. Early stages of sickle eye disease may not produce obvious vision changes; therefore, routine examinations are advised.

## Special Indications for Transfusion in Sickle Cell Patients

### Pregnancy

Pregnancy poses special risks to the mother with SCD and to her fetus. Potential maternal complications include an increased frequency of painful vasoocclusive crises, worsening of anemia, increased infection (especially urinary tract infections), toxemia,

and death. The fetus is at increased risk for growth retardation, premature birth, stillbirth, and spontaneous abortion. In mothers with HbSS disease, maternal complications and fetal death occur most frequently in the third trimester, whereas fetal death is more frequent in the first trimester in mothers with HbSC disease.<sup>66</sup> In 1971, Fort et al<sup>67</sup> reported an overall maternal death rate of more than 6% and an infant perinatal mortality rate of approximately 45% in mothers with SCD. These authors concluded that measures should be taken to prevent pregnancies in these women, including sterilization or abortion. Since 1972, the maternal death rate has been less than 2% and the perinatal death rate has been less than 5%.<sup>68</sup>

Recommendations for the prenatal care of the mother with SCD have included prophylactic transfusion at the onset of pregnancy or at the beginning of the third trimester, prophylactic transfusion only in those pregnancies considered high risk, or obstetric management without transfusion. Many of the earlier studies of transfusion in pregnant women with SCD are difficult to evaluate because of differences in design and the use of historical controls.

The most informative study to date is that of Koshy et al.<sup>69</sup> In this prospective study, 72 pregnant women with SCD were randomly assigned to receive either prophylactic RBC transfusions or only therapeutically indicated transfusions for defined medical or obstetric emergencies. Those patients assigned to the prophylactic transfusion study arm received an average of 12 RBC units during the course of pregnancy, while nearly one-half of the controls received an average of only 6.5 RBC units. When patients with high-risk factors (ie, multiple gestation or a history of previous perinatal mortality) were excluded, there were no significant differences in maternal and fetal outcome between the prophylactic transfusion and control groups, except (as might be expected) that the number of pain crises in the prophylactic transfusion group was significantly reduced. These findings suggest that prophylactic transfusion therapy may be unnecessary, and better avoided, in pregnant women with SCD who lack underlying high-risk factors.

Prophylactic transfusions have been advised for pregnancies in women with SCD who have high-risk features such as multiple gestation or history of perinatal loss.<sup>70</sup> Other indications for transfusion include toxemia, septicemia, acute renal failure, hypoxemia, and ACS.<sup>71</sup> It is important to recognize that even when high-risk features are absent, and prophylactic transfusions are avoided, between one-quarter and one-half of pregnant women with SCD will require RBC transfusion on an emergency basis because of major complications.<sup>70,72</sup> This should serve as a sobering reminder that all pregnant women with SCD require meticulous care.

### Surgery

The patient with SCD is at increased risk for perioperative complications including sudden death, pulmonary infarction, infection, and pain crisis. Changes in regional perfusion, accidents of anesthesia (eg, aspiration, difficulty in intubation, etc), or a variety of surgical circumstances may alter oxygenation, hydration,

temperature, or acid-base balance and lead to localized or generalized sickle cell vasoocclusion.

Some studies have reported favorable surgical results without the use of prophylactic transfusion. Homi et al<sup>73</sup> avoided transfusion throughout the entire perioperative period in 137 of 200 sickle cell patients who underwent surgery with general anesthesia. In the remaining patients transfusions were given either to restore the hemoglobin to baseline levels or to combat anticipated or actual blood loss. Six patients died, all during the postoperative period. Detailed transfusion information related to these fatalities was not provided, but five of the six patients had undergone emergency surgery and were in poor physical condition preoperatively. In a nonrandomized study of 66 patients with sickle syndromes (50 HbSS, 13 HbSC, 3 HbS/ $\beta$  thalassemia) who underwent 82 surgical procedures, no major differences in perioperative morbidity and mortality were seen, regardless of whether transfusions were given preoperatively, intraoperatively, postoperatively, or not at all.<sup>74</sup>

Other groups have routinely employed elective preoperative transfusion with good results. Janik and Seeler<sup>75</sup> described 32 patients with HbSS disease who underwent 46 operations following preparative RBC transfusions of 15 to 20 mL/kg, designed to increase the hematocrit to a minimum of 36%. The posttransfusion level of HbS was between 32% and 55%. In this series, there was no reported morbidity or mortality. Prophylactic exchange transfusions designed to achieve hematocrit values of more than 35% and HbA concentrations of more than 40% were administered in another study of 42 patients with SCD.<sup>76</sup> There were no reported deaths, and postoperative complications were infrequent and unremarkable; there were no apparent cases of vasoocclusive crisis or pulmonary embolus. Preoperative exchange transfusions have also been given successfully to patients with SCD before vitreoretinal surgery in order to diminish the risk of anterior segment ischemia.<sup>77</sup>

The Preoperative Transfusion in Sickle Cell Disease Study Group has provided the most comprehensive comparison of conservative and aggressive transfusion regimens. In 1995, this group reported results from a prospective study of 551 patients (a total of 604 operations), some of whom were randomly assigned to one of two transfusion regimens.<sup>78</sup> The aggressive transfusion regimen was intended to achieve a HbS concentration of 30% or less, while the conservative transfusion regimen sought to attain a minimum hemoglobin concentration of 10 g/dL. Although some overlap existed in the hematologic features of the two groups, data were analyzed on an intent-to-treat basis. Cholecystectomies, and otolaryngologic and orthopedic procedures accounted for more than three-fourths of the operations performed. A serious or life-threatening complication, most commonly ACS, was experienced by about 20% of the patients in each group. The aggressively transfused group used twice as many RBC units and had twice the alloimmunization rate, in comparison with the conservatively managed group.

The study group reported a subset of 364 patients who underwent cholecystectomy, using either traditional (58% of patients)

or laparoscopic techniques (42% of patients).<sup>79</sup> In this study, 110 patients were randomly assigned to receive aggressive preoperative transfusion, 120 patients were randomly assigned to receive conservative transfusion, 37 patients were nonrandomly assigned to receive no transfusion, and 97 patients were nonrandomly assigned to receive transfusion. Among all groups combined, the total complication rate was 39%, and sickle cell events occurred in 19%. However, patients who were nonrandomly assigned to receive no transfusion had a sickle cell complication rate of 32%. Patients who underwent laparoscopic procedures had longer anesthesia time but shorter duration of hospitalization, compared with those who had an open abdominal procedure. The incidence of complications did not differ between these two groups.

In a review of 118 patients who underwent elective tonsillectomy and adenoidectomy or myringotomy, and who were randomly assigned to either conservative or aggressive preoperative transfusion protocols, the study group found no major differences in complication rates.<sup>80</sup> About one-third of all patients experienced serious, nontransfusion-associated complications.

In all these reports, the study group concluded that a conservative preoperative transfusion regimen had equivalent efficacy to an aggressive transfusion regimen in preventing perioperative complications. Furthermore, the conservative transfusion regimen was associated with one-half the transfusion-associated complications. Therefore, a conservative preoperative transfusion approach was advocated. However, other interpretations of the data should be considered as well. For example, because of the overlap in percentage of HbS achieved between the aggressively and conservatively transfused groups, the two groups may not have differed from each other sufficiently to produce clinically distinct outcomes. Also, the aggressively transfused group may not have been transfused intensely enough to provide adequate physiologic protection from sickle cell events. The experience from Duke University supports the latter hypothesis: a very low complication rate was experienced by sickle cell patients there who were preoperatively transfused to mean hemoglobin values of 11.2 g/dL and an average of 21% HbS.<sup>81</sup>

Based upon all these results, the following guidelines would seem to be appropriate for most sickle cell patients who are undergoing surgery:

1. The most critical aspect of perioperative management is meticulous anesthetic and surgical care, avoiding hypoxemia, dehydration, acidosis, and hypothermia.
2. Preoperative RBC transfusions are warranted when prolonged general anesthesia is used or anticipated (ie, when there is reasonable likelihood that local anesthesia will prove to be inadequate). For uncomplicated minor procedures in which brief inhalation anesthetic is used (eg, some dental procedures), transfusion is seldom necessary.
3. The specific method of transfusion—regular or partial exchange—is not as important as the hematologic endpoint. A preoperative HbS of 30% to 40% and hemoglobin concentration in an intermediate range (10 to 12 g/dL) are desirable,

based upon empirical clinical experience and data cited previously. Special surgical situations characterized by low regional blood flow, especially if in a critical anatomic site (eg, major cardiac, orthopedic, or neurosurgery), may warrant even more aggressive preoperative transfusion. The choice of the method of transfusion largely depends upon the clinical circumstance. When surgery is urgent, a single-volume exchange transfusion (60 to 70 mL/kg) will rapidly achieve the hematologic goal, but when sufficient time is available before elective surgery, repeated regular transfusions over 2 to 4 weeks can be employed, with the attendant benefits of greater technical ease, lower procedural risk, and possibly a lower overall transfusion requirement.

4. In the various compound heterozygous sickle syndromes (with the exception of HbS/ $\beta^0$  thalassemia, which has a hemoglobin pattern similar to that of HbSS disease) it is safest not to monitor the percentage of HbS per se, but to consider the percentage of sicklable cells.

5. In most circumstances no special measures need to be taken in the preoperative management of patients with sickle cell trait. Patients with sickle cell trait probably do not even require transfusion before cardiopulmonary bypass.<sup>82</sup> However, prophylactic transfusion may well be appropriate for patients with sickle cell trait who will be undergoing orthopedic surgery requiring prolonged tourniquet application, although this view is not universally held.<sup>83</sup>

### Radiologic Imaging

Cerebral or coronary angiography in patients with SCD should be performed only after prophylactic transfusion has achieved a HbS concentration of less than 20% and with adequate hydration.<sup>84</sup> Magnetic resonance imaging (MRI) and magnetic resonance angiography (MRA) have largely supplanted catheter angiography for neuroimaging in SCD, and can be safely performed using gadolinium contrast without concern for inducing vasoocclusion.<sup>85</sup> Intravenous urography may be performed without preparative transfusion, but vigorous hydration should be maintained both before and after the procedure.

## Donor Selection and Transfusion-Related Complications in Patients with Sickle Cell Disease

### Alloimmunization

Sensitization to transfused red cell antigens (alloimmunization) may lead to an inconvenient and costly delay or even a life-threatening inability to find compatible blood. In addition, significant alloimmunization in patients with SCD may result in delayed hemolytic transfusion reactions (DHTRs) that are often serious and occasionally even lethal. Whenever there is a prolonged or repeated requirement for transfusion, it is prudent to obtain a full red cell antigen profile (including, for example, C, E, K, Fy<sup>a</sup>, and Jk<sup>b</sup> phenotypes) of the recipient before transfusion therapy is initiated. This information will be very useful later if alloimmunization is suspected.

Studies of adult and pediatric patients who have been transfused suggest that the overall risk of alloimmunization in SCD is

about 20% to 30%. Many of these studies, however, were retrospective and did not examine the patients' transfusion histories before the study.

Patients who are frequently transfused appear to be somewhat more likely to develop alloantibodies than those who receive infrequent transfusions. Chronically transfused patients who develop alloantibodies often appear to do so early in their transfusion courses<sup>86</sup> and are also more likely to develop multiple alloantibodies. In this regard, patients with SCD appear to be similar to the population at large: there is a subpopulation of "hyperresponders" who readily become alloimmunized, but the majority are nonresponders in spite of numerous transfusions.<sup>87</sup> Approximately one-half to two-thirds of alloantibodies identified in patients with HbSS disease have been directed at antigens of the Rh blood group system, and the majority of these are anti-Rh(E). Anti-Kell (K) and anti-Kidd (Jk<sup>a</sup> and Jk<sup>b</sup>) account for approximately 20% of alloantibodies in transfused patients with SCD.<sup>87</sup> Lewis antibodies account for up to one-third of alloantibodies, but as in the general population these are generally regarded as "naturally occurring" and do not appear to be clinically significant.

The impact of racial differences between donors and recipients on the rates of alloimmunization of patients with HbSS disease has been examined in several studies. Overall, approximately 20% to 30% of transfused patients with SCD become alloimmunized, in comparison with 5% to 10% of thalassemia patients who are transfused in the United States.<sup>88</sup> The increased incidence of alloimmunization in patients with SCD has been attributed to the greater disparity in race and red cell phenotypic profile that exists between typical blood donors and sickle cell recipients, in comparison with other recipients.<sup>88,89</sup> In many urban centers, often 90% to 95% of blood donors are of European ancestry<sup>88,89</sup> and often possess a variety of Rh, Duffy, Kell, and Kidd antigens that are less frequently found in recipients of African ancestry.<sup>90</sup> Orlina et al<sup>89</sup> calculated that a Black recipient has a 33% chance of compatibility for these antigens from a Black donor pool, in comparison with an only 3% chance of compatibility with the typical urban donor pool of 90% White and 10% Black donors. However, 70% to 80% of Black populations are Fy(a-b-), whereas more than 99% of White populations are positive for one or both antigens, yet the frequency of Duffy alloimmunization in transfused sickle cell patients is only 15% or less.<sup>87</sup> When the blood donor and recipient belong to different racial groups, the risk of alloimmunization depends not only on whether the two populations have different frequencies of blood group antigens but also on the intrinsic antigenicity of each blood group antigen.

Previous recommendations have included selecting donors 1) according to an extended red cell antigen profile, so that compatibility is achieved for Kell and secondary Rh group antigens (ie, C and E), 2) according to race, and 3) according to no special criteria other than the standard ABO group and Rh(D) type.

If any special preselection of donors is performed, it should be maintained on a regular basis, because hyperresponders often

become immunized after only 10 to 20 transfusions. Under most circumstances, it is not cost effective or feasible to pre-select donors for sickle cell patients. Routine donors are usually acceptable. If a patient with SCD has clinically significant alloantibodies, then specific compatibility testing must, of course, be performed. Because of the existence of hyperresponders, it is advisable to match as closely as possible all individuals who have developed one or more alloantibodies. In such cases, the search for compatible blood is hastened by screening units obtained from same-race donors.<sup>88</sup>

### ***Delayed Hemolytic Transfusion Reaction***

This complication is caused by acute destruction of transfused red cells beginning several days after transfusion. It is typically caused by an alloantibody to a blood group antigen produced in anamnestic fashion in a previously sensitized patient. Clinically, delayed hemolytic transfusion reaction (DHTR) is characterized by fever and a precipitous decrease in hemoglobin, often to levels lower than those that existed before transfusion. Hemoglobinuria is often seen, resulting from intravascular hemolysis. DHTR has distinctive features when present in patients with SCD, and thus it may even constitute a unique syndrome in this setting.<sup>91</sup> It occurs in about 5% to 20% of regularly transfused patients with SCD, an incidence considerably higher than that seen in other multitransfused populations.<sup>92</sup> Pain is commonly experienced by sickle cell patients with DHTR and it may be readily confused with that from vasoocclusive crisis. Marked reticulocytopenia is occasionally seen, which contributes to the anemia. When DHTR is suspected, serial hemoglobin fractionation may be a useful means to document alloimmune hemolysis, by showing a rapid fall in donor-cell-derived HbA. However, although hemolysis of donor red cells is typically the principal concern, destruction of autologous cells can occur as well through a phenomenon that has been referred to as “bystander hemolysis.”<sup>93</sup> In this process, antigen-negative red cells are destroyed by autoantibodies with an indeterminate specificity, or by deposition of antigen-antibody complexes on red cell membranes, causing complement activation and lysis of red cells. These reactions are an especially serious threat, because this potentially life-threatening complication is not always prevented by the use of phenotypically matched blood. Corticosteroids, intravenous immune globulin, and erythropoietin have been used to treat this complication. In the face of a DHTR, additional transfusion should be avoided whenever possible in order to prevent exacerbation of the hemolytic process.

### ***Blood from Sickle Cell Trait Donors for Patients without Sickle Cell Disease***

The oxygen-carrying capacity of HbAS blood is comparable to that of normal (HbAA) blood, and in the great majority of general, elective transfusion settings, HbAS blood may be transfused without any untoward consequence. However, this blood should be avoided in neonates (especially ventilator-dependent premature infants), in patients with severe cardiopulmonary illness or shock, and in those who require high-volume

blood transfusions (eg, in cases of severe acute hemorrhage). All of these conditions are sometimes associated with hypoxemia and acidosis severe enough to cause sickling of sickle trait erythrocytes. Leukocyte reduction filters often are unsuccessful, and HbAS erythrocytes sometimes hemolyze during the process of deglycerolization.<sup>94,95</sup> *Accordingly, RBC units destined for leukocyte reduction or frozen storage should be screened to detect, and possibly eliminate, units containing sickle cell trait.*

Because HbAS blood is acceptable for routine transfusion to individuals without sickle cell disease, there should be no objection to using autologous transfusion in patients with sickle cell trait, as long as none of the risk factors cited above is present.<sup>96</sup>

### ***Blood from Sickle Cell Trait Donors for Patients with Sickle Cell Disease***

Blood obtained from donors with sickle cell trait is undesirable for use in patients with SCD, primarily because the resultant mixture of HbSS and HbAS cells will obfuscate hemoglobin fractionation results that are generally used to monitor the progress of transfusion therapy. In addition, in nearly all sickle cell conditions requiring transfusion therapy, there is a danger of progressive sickling. As noted above, even HbAS blood can sickle under severely unfavorable physiologic conditions and thus should be avoided.

## **Thalassemias**

### ***Epidemiology***

The frequency of thalassemia genes in world populations is surprisingly high, with the thalassemias in aggregate representing the most prevalent of all known genetic diseases.<sup>97</sup> Occasional individuals with thalassemia have been observed in native populations in virtually all areas of the world where they have been sought, but most cases are concentrated within a broad subtropical “thalassemia belt” extending from Western Europe to Southeast Asia (Fig 29-1). Within this region there are areas with especially high frequencies of thalassemia genes, with as many as 50% of some populations having one or more forms of thalassemia.

The characterization in recent years of the underlying mutations of the thalassemia syndromes has added considerably to our understanding of the population genetics of these disorders. In general, each of the geographic areas with a high prevalence of thalassemia, particularly the  $\beta$  form, has a characteristic and often unique group of thalassemia mutations. In most of these populations, a small number of mutations account for most of the observed cases. These mutations probably arose independently in each population, and they achieved high frequency because of strong selective pressure. There is considerable information suggesting that resistance to *P. falciparum* malaria is the selective force responsible for the high frequency of thalassemia. One line of evidence in support of this hypothesis is the geographic concurrence of the “thalassemia belt” and the areas where malaria was formerly endemic (Fig 29-1).

Molecular Basis for the Thalassemia Syndromes

More than 200 distinct thalassemia mutations are now known. A summary of the major categories of these molecular abnormalities is shown in Table 29-3; a detailed description is given elsewhere.<sup>1(pp194-195)</sup> In spite of the considerable diversity of forms and sites of mutations in the globin genes, as well as the variety of mechanisms by which they lead to inhibition of globin-chain synthesis, the clinical phenotypes of most forms of thalassemia are similar (see below). For most clinical purposes, therefore, the identification of the specific mutations of thalassemia patients is of little importance. However, for prenatal diagnosis, and because of the potential for ameliorating the effects of thalassemia genes, this information may eventually assume fundamental significance.

Pathophysiology

In most of the symptomatic forms of thalassemia, the pathophysiology follows the same mechanism. The pathogenesis of homozygous  $\beta$ -thalassemia (“Cooley anemia”) serves as a representative example.

Marrow erythroid cells of affected individuals exhibit a diminution or absence of hemoglobin  $\beta$ -chain synthesis. Although this defect is partially compensated for by an increase in the synthesis of HbF  $\gamma$  chains, a major disparity nevertheless exists between  $\alpha$ -chain and non- $\alpha$ -chain synthesis (Fig 29-6), with  $\alpha$ -chain synthesis occurring in considerable excess. The deficiency of  $\beta$  and  $\gamma$  chains results in severe underhemoglobinization of the red cells because of insufficient quantities of HbA ( $\alpha_2\beta_2$ ) or HbF ( $\alpha_2\gamma_2$ ).

The excess of uncombined  $\alpha$  chains that accumulate in the red cells of these patients is also responsible for other deleterious effects. Uncombined  $\alpha$  subunits are unstable and precipitate within erythrocytes. This process, which is accompanied by oxidation of heme iron, releases superoxide ions, which in turn inflict oxidative damage on intracellular, and particularly membrane, components of the red cell.<sup>98,99</sup> In severe forms of  $\beta$ -thalassemia, the latter process results in destruction of a major fraction of erythroid cells, even before they are released from the marrow, producing what has been termed “ineffective erythropoiesis.” In compensation for this process, these patients

develop an enormous expansion of their marrow, a change that produces characteristic bony deformities most apparent in the skull and face. The resulting anemia in the most severe forms of homozygous  $\beta$ -thalassemia ( $\beta^0$ ) is incompatible with long-term survival unless periodic transfusions are given.

Compound Heterozygous Forms

In geographic areas with high frequencies of thalassemia, both  $\alpha$ - and  $\beta$ -thalassemia alleles may coexist in the same population, and both types of abnormalities may therefore occur in the same individual. The combination of homozygous  $\beta^0$ -thalassemia with concomitant  $\alpha$ -thalassemia produces a clinically mild form of

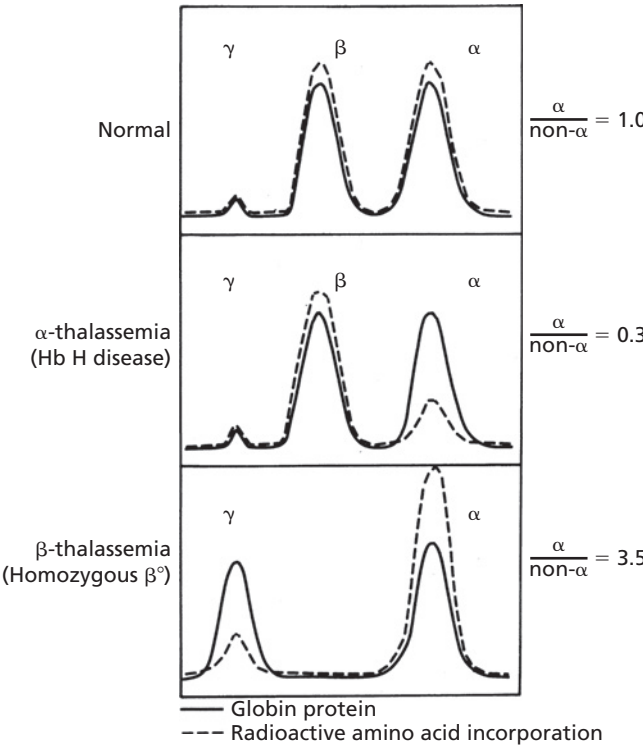


**Figure 29-6.** Synthesis of globin chains by erythroid cells from a normal individual (top), a patient with  $\alpha$ -thalassemia (middle), and a patient with severe  $\beta^0$ -thalassemia (bottom).<sup>1(p192)</sup>

**Table 29-3.** Examples of Molecular Abnormalities of Thalassemia Genes

| Abnormality                                                             | Consequence                                                                        |
|-------------------------------------------------------------------------|------------------------------------------------------------------------------------|
| Globin-gene deletions, partial or complete                              | Absence of globin synthesis; formation of fusion genes; elevated expression of HbF |
| Nucleotide substitutions in the promoter regions                        | Inhibition of globin-gene transcription                                            |
| Nucleotide substitutions or deletions at splice-junction sites          | Prevention of the formation of normal globin messenger RNA (mRNA)                  |
| Nucleotide substitutions in the coding segments or introns              | Abnormally spliced globin mRNA                                                     |
| Nucleotide substitutions in the transcription-termination signal region | Formation of abnormal globin mRNA                                                  |
| Nucleotide substitutions in translation-initiation codons               | Inhibition of mRNA translation                                                     |
| Nucleotide deletions or insertions                                      | Formation of premature termination codons                                          |
| Nucleotide substitutions in the translation-termination codons          | Production of extended-length globin chains                                        |
| Mutations that produce highly unstable globin chains                    | Rapid degradation of globin chains within the erythrocytes                         |

Cooley anemia,<sup>100</sup> undoubtedly because a more nearly normal balance is achieved between  $\alpha$ -chain and  $\beta$ -chain synthesis, so that the harmful series of events described above is attenuated.

Hemoglobin E, an abnormal hemoglobin prevalent in Southeast Asia, produces thalassemia-like hematologic effects, possibly by two separate mechanisms. First, HbE is an unstable variant and may undergo  $\beta$ -chain loss by intracellular precipitation or degradation or both. Second, the HbE mutation produces an aberrant splicing site, leading to a decreased production of  $\beta^E$  messenger RNA (mRNA).<sup>101</sup> Large series of patients with the compound heterozygous combination of HbE and  $\beta^0$  thalassemia have been reported.<sup>102</sup> This syndrome has variable expression, with more severely affected individuals requiring periodic transfusions to maintain adequate hemoglobin levels.

### Homozygous $\beta$ -Thalassemia (Cooley Anemia)

#### Clinical Features and Natural History

The clinical and hematologic features of the major forms of  $\beta$ -thalassemia are summarized in Table 29-4. Because the molecular abnormalities that cause these disorders are within, or in close proximity to, the  $\beta$ -globin genes, the onset of expression of  $\beta$ -thalassemia occurs some months after birth when HbF is replaced by HbA. Affected infants are therefore normal at birth, and evidence of the disease first appears by the middle or end of the first year. Anemia gradually develops, accompanied by progressive enlargement of the liver and spleen and cardiovascular changes related to congestive heart failure. A large fraction of the cardiac output is shunted through the hypertrophied marrow of untransfused thalassemia patients and this change contributes to an expansion of the plasma volume, resulting in increasing

hemodilution and worsening cardiac failure. Before the introduction of modern transfusion methods, the size of the spleen and the liver in these patients progressed to a massive degree, primarily because of reticuloendothelial cell proliferation. Hyperexpanded marrow also results in a thinning of cortical bone, predisposing these children to fractures. Craniofacial bone deformities are variable in degree but often severe, frequently resulting in considerable malocclusion caused by maxillary hyperplasia. In the absence of transfusion support, children with severe  $\beta$ -thalassemia succumb to the effects of anemia and heart failure within the first years of life. Milder forms of  $\beta$ -thalassemia are accompanied by a slower rate of progression and correspondingly longer survival.

#### Transfusion Therapy for Patients with $\beta$ -Thalassemia

The earliest transfusion regimens for patients with  $\beta$ -thalassemia consisted of administering mainly whole blood as infrequently as possible and only when severe anemia developed.<sup>102</sup> This form of management protected these children from early death from progressive heart failure but did not mitigate the full expression of the disease. A critical turning point in the approach to the transfusion management of  $\beta$ -thalassemia patients came in 1964, when reports by Schorr and Radel<sup>103</sup> and Wolman<sup>104</sup> compared the clinical status of patients whose hemoglobin levels had been maintained at different levels. The principal finding was that children with thalassemia fared better if their hemoglobin levels were maintained above 8 g/dL. Compared with other  $\beta$ -thalassemia patients, the group with a high hemoglobin level exhibited more normal growth, a lesser extent of liver and spleen enlargement, less bony deformity and orthodontic abnormalities,

**Table 29-4.** Clinical and Hematologic Features of the Major Forms of  $\beta$  Thalassemia

| Type                             | Hemoglobin Findings                                                                 | Hematologic Changes                                                                                         | Clinical Features                                                                                          |
|----------------------------------|-------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------|
| <b>Heterozygous</b>              |                                                                                     |                                                                                                             |                                                                                                            |
| $\beta^0$ (severe) (high $A_2$ ) | $A_2$ , 3.5-7.5% F, 1-6%                                                            | Erythrocyte microcytosis and hypochromia; mild to moderate anemia                                           | Possible splenomegaly and mild icterus                                                                     |
| $\beta^+$ (mild) (high $A_2$ )   | $A_2$ , 3.5-7.5%                                                                    | Erythrocyte microcytosis and hypochromia; mild or absent anemia                                             | Usually none                                                                                               |
| $\beta$ silent carrier           | $A_2$ and F, normal (F-containing cells sometimes detectable by slide elution test) | Hematologically normal                                                                                      | None                                                                                                       |
| $\delta\beta$ (high F)           | $A_2$ , normal or low F, 5-20%                                                      | Erythrocyte microcytosis and hypochromia; mild or absent anemia                                             | Usually none                                                                                               |
| <b>Homozygous</b>                |                                                                                     |                                                                                                             |                                                                                                            |
| $\beta^+$                        | F, 30-95%                                                                           | Markedly abnormal red cell morphology with microcytosis and hypochromia, nucleated red cells; severe anemia | Pallor, jaundice, bone deformities with abnormal facies, hepatosplenomegaly, usually transfusion-dependent |
| $\beta^-$                        | F, 40-80%                                                                           | Poikilocytosis, anisocytosis, target cells; moderate anemia                                                 | Pallor, hepatosplenomegaly, jaundice; transfusions not usually required                                    |
| $\delta\beta$ (high F)           | F, 100%                                                                             | Poikilocytosis, anisocytosis, hypochromia, microcytosis; mild to moderate anemia                            | Mild jaundice, hepatosplenomegaly usually present                                                          |

and a considerably lower frequency of cardiac enlargement. Although these patients had received a larger number of transfusions and had correspondingly higher levels of iron stores, their considerably improved clinical status demonstrated that, at least in younger children, the hemoglobin level was more important than iron overload as a determinant of clinical status.<sup>104</sup>

The findings from these studies led rapidly to the introduction of transfusion regimens that sought to maintain higher hemoglobin levels, usually higher than 9.5 or 10 g/dL. This approach, frequently referred to as “hypertransfusion,” usually consists of RBC transfusions of about 20 mL/kg, given whenever the patient’s hemoglobin falls below the targeted level. Once these patients are fully transfused, the requirement to maintain them on the hypertransfusion regimen is no greater than that needed to maintain patients at a lower hemoglobin level.<sup>105</sup> Immediate posttransfusion hemoglobin levels typically average 14 g/dL, returning to baseline after 3 to 6 weeks. Hypertransfusion is also accompanied by a reduction in gastrointestinal absorbance of iron.<sup>106</sup> The rate of iron accumulation can therefore be lessened while maintaining a more desirable hemoglobin level.

In addition to improved clinical status, the correction of anemia by hypertransfusion also exerts a suppressive effect on the erythropoietic drive that causes marrow hypertrophy. The bony abnormalities, which in the past were so characteristic of this disease, are now entirely preventable,<sup>107</sup> and these patients now experience no increased risk of fractures. In addition, the marrow suppressive effect of hypertransfusion prevents the release of the deformed, short-lived Cooley anemia erythrocytes and delays the onset of hypersplenic changes, permitting splenectomy to be postponed safely until an older age.

Appropriately transfused patients with Cooley anemia gradually experience increasing splenomegaly caused by reticuloendothelial proliferation. Although this process is considerably delayed by hypertransfusion, it cannot be totally prevented. The development of hypersplenism shortens the transfusion interval and increases transfusion requirements. Piomelli and Loew<sup>108</sup> have suggested that RBC transfusion of more than 200 mL/kg per year in these patients is usually an indication for splenectomy.

Propper et al<sup>109</sup> have proposed a transfusion regimen for Cooley anemia that maintains the hematocrit above 35%, but this “supertransfusion” approach is no longer recommended.<sup>97</sup> Indeed, moderate transfusion regimens that target a baseline hemoglobin level of 9 to 10 g/dL appear to offer the best combination of preventing the long-term adverse consequences of anemia and excessive erythropoiesis, while minimizing iron accumulation.<sup>110</sup>

Individuals with heterozygous  $\beta$ -thalassemia ( $\beta$ -thalassemia trait) ordinarily have no special need for transfusions. An exception is during pregnancy, when women with thalassemia trait often develop more severe anemia<sup>111</sup> and occasionally require transfusion support.<sup>112</sup> Those who have inherited a combination of both mild and more severe  $\beta$ -thalassemia mutations, termed thalassemia intermedia, exhibit a variable clinical course; some experience a mild clinical course that rarely requires intervention,

while others may require regular RBC transfusions, at least for some period.<sup>113</sup>

### $\alpha$ -Thalassemia

Wasi et al,<sup>102</sup> in summarizing their experience in Thailand with a large number of patients with HbH disease (Table 29-5), showed that this syndrome often produced Cooley anemia-like changes, with anemia, bony deformities, and splenomegaly. However, these changes appear to reflect, in part, the effects of infections and other environmental factors superimposed upon the genetic abnormality. HbH disease in the United States generally exhibits a relatively mild thalassemia-intermedia-like syndrome and seldom requires blood transfusion except during surgery.

The hydrops fetalis form of  $\alpha$ -thalassemia, in which  $\alpha$ -chain synthesis is totally lacking, has until recent years been invariably fatal, the infants surviving no longer than a few hours after birth.

A few recent examples have been reported of such infants who received vigorous resuscitative measures, including transfusions at birth, with survival beyond the neonatal period.<sup>114</sup> This syndrome is characterized by the production almost exclusively of homotetramers of  $\gamma$  and  $\beta$  chains (Table 29-5). These abnormal hemoglobins have virtually no physiologically useful oxygen-transporting capacity because of their very high oxygen affinity. Infants who survive are therefore at great risk of developing hypoxic encephalopathy during the neonatal period and are totally dependent on transfused RBC units throughout their lives.

### Effects and Management of Chronic Transfusion Therapy Hemosiderosis

Each RBC unit contains approximately 250 mg of iron. Unfortunately, physiologic mechanisms for the elimination of excess iron are lacking. Signs of clinical toxicity often become apparent when total body iron reaches 400 to 1000 mg/kg of body weight, and levels in excess of this are potentially lethal.<sup>115</sup>

Once reticuloendothelial sites of iron storage become saturated, parenchymal deposition increases and tissue damage ensues. The primary targets of iron toxicity are the heart, liver, pancreas, and other endocrine organs. Hepatotoxicity, expressed initially by fibrosis and subsequently cirrhosis, is the most common early manifestation of transfusion-related iron overload. Cardiac toxicity, causing cardiomyopathy and arrhythmia, has been the most frequent cause of death in chronically transfused patients.

The most practical measurement of body iron stores is the serum ferritin concentration, which is in equilibrium with storage iron up to a level of approximately 4000  $\mu$ g/L.<sup>115</sup> However, in the face of a high total body iron burden, and in a variety of disease states (eg, acute inflammatory conditions, hepatocellular damage), the reliability of serum ferritin as a marker for the total body iron burden diminishes. Serum ferritin is probably even less reliable as a marker of total body iron in patients with SCD.<sup>116</sup> The hepatic iron concentration determined from liver biopsy specimens is an excellent indicator of total body iron stores in

**Table 29-5.** Clinical and Hematologic Features of  $\alpha$  Thalassemia

| Form of $\alpha$ Thalassemia | Genotype                               | Hemoglobin Findings                                                                       |                                                          | Hematologic Changes                                                                                                                      | Clinical Features                                                                                                                                             |
|------------------------------|----------------------------------------|-------------------------------------------------------------------------------------------|----------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                              |                                        | Newborn                                                                                   | Adults                                                   |                                                                                                                                          |                                                                                                                                                               |
| Silent carrier               | $\alpha\alpha/\alpha-$                 | 1-2% Hb $\gamma_4$ (Bart's)                                                               | No abnormality                                           | Usually no abnormality; mild microcytosis may be present                                                                                 | Clinically normal                                                                                                                                             |
| $\alpha$ -Thalassemia trait  | $\alpha\alpha/-$ or $\alpha-/-\alpha-$ | 2-10% Hb $\gamma_4$                                                                       | No abnormality                                           | Red cell microcytosis and hypochromia with mildly abnormal morphology; anemia mild or absent                                             | Usually normal                                                                                                                                                |
| HbH disease                  | $\alpha-/-$                            | 20-30% Hb $\gamma_4$                                                                      | 5-25% Hb H ( $\beta_4$ ); may be traces of Hb $\gamma_4$ | Moderately severe anemia with marked anisopoikilocytosis and microcytosis; red cell inclusion bodies demonstrable by supravital staining | Pallor, jaundice<br>hepatosplenomegaly;<br>cholelithiasis occurs commonly in adults                                                                           |
| Hydrops fetalis              | $-/-$                                  | 80% Hb $\gamma_4$ , with the remainder Hb $\beta_4$ and Hb Portland ( $\zeta_2\gamma_2$ ) | —                                                        | Severe anemia, markedly abnormal erythrocyte morphology with anisopoikilocytosis, hypochromia, and pronounced erythroblastemia           | Massive<br>hepatosplenomegaly,<br>generalized edema with ascites and pleural and pericardial effusion;<br>nearly all are stillborn or die shortly after birth |

patients with thalassemia major.<sup>116</sup> Despite the relative difficulty in obtaining such specimens, this method is recommended for the reliable measurement of the total amount iron stored in the body.<sup>117</sup> A superconducting quantum interference device susceptometer measures body iron noninvasively and produces results equivalent to those obtained by liver biopsy, but this method is available in just a few centers worldwide.<sup>116</sup> MRI has been used to assess levels of iron in the liver, has recently been standardized, and may soon become the optimal method for the indirect measurement of iron in the liver.<sup>118</sup> MRI measurement of iron in the heart has also been studied, but this technique has not been fully standardized.<sup>116,117</sup>

When faithfully administered, treatment with daily subcutaneous deferoxamine has been shown to arrest or even reverse hepatic<sup>119</sup> and cardiac<sup>120</sup> iron toxicity in thalassemia patients, especially when started early in the course of transfusion therapy. This drug has also been effective in patients with sickle cell anemia.<sup>121</sup> Deferoxamine is usually prescribed in a daily dose of 50 to 75 mg/kg of body weight, administered subcutaneously over 8 to 12 hours (usually overnight) via a portable infusion pump. Because of the parenteral route of administration and the special measures required to use the pump, this agent is cumbersome to administer. The infusion may also be painful, especially for children. Not surprisingly, compliance is often poor, thus limiting the effectiveness of this therapy.

Oral iron chelators have been developed that offer the promise of far greater ease of administration, and hopefully, improved compliance. Deferiprone has been studied for over 20 years, and is currently available in Europe. Concerns were initially raised that deferiprone could be linked to an increased incidence of hepatic fibrosis, but further studies appear to have relieved that concern. Its main toxicity appears to be agranulocytosis, and so patients

receiving this medication should undergo frequent blood counts. Deferiprone may have only partial efficacy in reducing liver iron concentrations, but it actually might be superior to deferoxamine in lowering cardiac iron.<sup>116</sup> Deferasirox is the first oral iron chelator licensed in the United States, and is almost exclusively excreted in the bile, in contrast to deferoxamine and deferasirox, which are primarily excreted in the urine. Deferasirox is generally well tolerated, but it may cause nephrotoxicity. Studies to date have demonstrated that deferasirox is effective in reducing liver iron content, while only preliminary data show that it can lower cardiac iron. Both oral iron chelators, deferiprone and deferasirox, appear to be more effective than deferoxamine in binding to intracellular iron, while deferoxamine may have an advantage in chelating extracellular iron. Attempts have been made to combine therapy with both deferoxamine and either deferiprone or deferasirox. Early data seem to suggest that this type of combination indeed offers some additive benefit.

The transfusion regimen employed for the long-term management of chronically transfused patients can significantly affect the rate of iron accumulation. In sickle cell patients who require protracted courses of transfusion, exchange procedures can considerably decrease the rate of accumulation.<sup>122</sup>

## Erythrocyte Enzyme Abnormalities

Enzyme-mediated metabolic pathways in the red cell function to maintain the hemoglobin and the cell membrane in a reduced state. These objectives are accomplished primarily through the reactions linked to the pentose phosphate shunt and, for metabolic energy requirements, through the glycolytic pathway. Many enzyme deficiencies in these metabolic pathways have



been described. The majority are inherited as autosomal recessive abnormalities. Two exceptions—glucose-6-phosphate dehydrogenase (G6PD) deficiency and phosphoglycerate kinase deficiency—are transmitted in an X-linked recessive mode.

G6PD deficiency is prevalent in the same geographic areas in which there is a high frequency of hemoglobin disorders. In common with many of the hemoglobinopathies and thalassemia syndromes, hemizygous carriers of G6PD abnormalities are more resistant to *P. falciparum* malaria. Two common clinical forms of G6PD deficiency are the African variant, designated Gd<sup>A</sup>, and the Mediterranean variant, Gd<sup>Mediterranean</sup>. Both are characterized by an accelerated age-dependent decrease in red cell G6PD activity. In the cells of affected males or homozygous females with the African Gd<sup>A</sup> variant, there is a comparatively slow decline in enzyme activity with age, so that only a minority of cells, the most senescent, are substantially lacking in enzyme activity. The Gd<sup>Mediterranean</sup> variant has much less enzyme activity, and in affected males even newly released reticulocytes have a substantial deficiency in enzyme activity; the mature circulating erythrocytes are essentially devoid of measurable G6PD activity.

G6PD catalyzes the initial step in the pentose phosphate shunt pathway, which is essential in the mature red cell for the regeneration of reduced nicotinamide adenine dinucleotide phosphate (NADPH) from nicotinamide adenine dinucleotide phosphate (NADP). The reduced form is a required cofactor for the enzyme glutathione reductase, which together with glutathione peroxidase protects the hemoglobin, the supporting structures, and the enzymes of the red cell against oxidative damage. In the face of G6PD deficiency, erythrocytes may be extremely vulnerable to oxidant stress, resulting from exposure to certain drugs, chemicals, and infectious agents. During periods of oxidant stress, the hemoglobin may undergo intracellular precipitation to form Heinz bodies. These precipitates, which become associated with the cell membrane, are normally removed in the spleen, but in the process the cell may become damaged and undergo rapid destruction.

Both the African and the Mediterranean types of G6PD deficiency are associated with little or no hematologic abnormality under normal circumstances. In individuals with the African variant, only a comparatively small fraction of circulating red cells are significantly deficient in G6PD at any given time, and even though severe hemolysis and anemia may result from an episode of oxidative stress, the process is typically self-limited. The younger population of red cells that remains after hemolysis has occurred retains a higher level of enzyme activity, and these cells are thus relatively protected from further oxidative challenge. In individuals with the Mediterranean variant, all of the circulating erythrocytes are severely G6PD deficient and hence susceptible to oxidant challenge. Patients with this variant are at risk for acute, life-threatening hemolysis if exposed to a significant oxidant challenge. Transfusions are often lifesaving during episodes of acute hemolysis.

Pyruvate kinase (PK) deficiency occurs most often in northern Europeans and is the most prevalent enzyme deficiency

disorder affecting the glycolytic pathway. Impaired synthesis of adenosine triphosphate in PK-deficient red cells results in potassium depletion, intracellular dehydration, and impaired passage of the erythrocytes through the spleen. Premature destruction ensues in the spleen, liver, or marrow, resulting in variable degrees of anemia. In severe forms of the disease, splenectomy may ameliorate the degree of hemolysis, but ongoing transfusion support may nevertheless be required to maintain an adequate hemoglobin level in these patients.

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## Disorders of the Red Cell Membrane

The primary functions of the red cell membrane are to maintain the structural integrity of the cell and to regulate cation transport and permeability. The most prevalent of the red cell membrane disorders include hereditary spherocytosis (HS) and hereditary elliptocytosis (HE). Rarer membrane-related disorders include a group of hereditary stomatocytosis syndromes and hereditary pyropoikilocytosis. Hereditary spherocytosis predominates in individuals of northern European extraction, whereas HE has been found in numerous population groups. There are numerous variants of each of these disorders, and in most cases inheritance follows an autosomal dominant pattern.

The extent of hemolysis in HS and HE varies considerably among the different variants. In some cases hemolysis is fully compensated and barely perceptible, whereas in more severe forms anemia may be pronounced. The underlying defects in these disorders are structural abnormalities of the red cell membrane cytoskeleton, but this alone is not the direct cause of hemolysis. Rather, the misshapen cells have impaired deformability and difficulty in traversing the splenic circulation, and undergo destruction within the spleen. Accordingly, splenectomy is frequently effective in ameliorating the hemolysis and the anemia, especially in HS.

Before splenectomy, patients with these hemolytic disorders are also at risk of developing acute episodes of aplasia accompanied by reticulocytopenia and a rapid decrease in hemoglobin levels. These episodes typically follow a trivial viral illness, and they have been linked to the same parvovirus agent that causes aplastic episodes in patients with SCD.<sup>25</sup> During these aplastic crises, as in sickle cell disease, RBC transfusions may be required to sustain life.

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## Acknowledgment

The author gratefully acknowledges the contributions of Dr. George R. Honig, who co-authored previous editions of this chapter.

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## Disclaimer

The author has disclosed no conflicts of interest.

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Many aspects of hematopoiesis are either incompletely developed in preterm infants or are adapted to serve the fetus (ie, the intrauterine counterpart to a live-born preterm neonate). This lack of development and/or adaptation to extrauterine life diminishes the capacity of the neonate to produce red cells, platelets, and neutrophils—particularly during the stress of life-threatening illnesses encountered after preterm birth such as sepsis, severe pulmonary dysfunction, necrotizing enterocolitis, and immune cytopenias. Similarly, hepatic function is immature, and the result is low levels of plasma clotting proteins. The serious medical and surgical problems of preterm birth can be further complicated by phlebotomy blood losses, bleeding, hemolysis, and consumptive coagulopathy. Thus, preterm infants begin life with quantities of blood cells and clotting proteins that are barely adequate. Furthermore, these infants have a diminished ability to increase production adequately to compensate for the hematologic problems they experience. These circumstances lead to the need for blood component transfusions.

Preterm infants, especially those with a birthweight less than 1.0 kg and with respiratory distress, are given numerous red cell transfusions early in life because of several interacting factors. Neonates delivered before 28 weeks of gestation (birthweight <1.0 kg) are born before the bulk of iron transport has occurred from mother to fetus via the placenta and before the onset of marked erythropoietic activity of fetal marrow during the third trimester. Hence, preterm infants of very low birthweight enter extrauterine life with low iron stores and a small circulating volume mass of red cells. Soon after preterm birth, severe respiratory disease can lead to repeated blood sampling for laboratory studies and, consequently, to replacement red cell transfusions. As a final factor, preterm infants are unable to mount an effective erythropoietin (EPO) response to decreasing numbers of red cells, and this factor contributes to the diminished ability to compensate for anemia and enhances the need for transfusions.

The physiology of hematopoiesis in the fetus and newborn is discussed in detail in Chapter 25, hemolytic disease of the fetus and newborn in Chapters 26 and 27, congenital hemolytic anemias in Chapter 29, and the physiology and disorders of plasma clotting and anticoagulant proteins in Chapter 28. Accordingly, only the aspects of physiology and pathophysiology that pertain to neonatal transfusion medicine, in general, are included here. The emphasis is on transfusion management during the first several weeks after birth.

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## Red Cell Transfusion

### Pathophysiology of Anemia of Prematurity

During the first weeks of life, all infants experience a decline in the number of circulating red cells caused by physiologic factors. In sick preterm infants, phlebotomy blood losses also contribute to the decline. In healthy term infants, the nadir blood hemoglobin value rarely decreases to less than 9 g/dL (mean = 11 to 12 g/dL) at an age of approximately 10 to 12 weeks.<sup>1</sup> Because this postnatal decrease in hemoglobin level is universal and is well tolerated by term infants, it is commonly called the *physiologic anemia of infancy*. Among preterm infants, this decline occurs at an earlier age and is more pronounced in severity. Mean hemoglobin concentration decreases to approximately 8 g/dL in infants of 1.0 to 1.5 kg birthweight and to 7 g/dL in infants weighing less than 1.0 kg.<sup>2</sup> In preterm infants, this marked decline in hemoglobin frequently is exacerbated by phlebotomy blood losses and may be associated with symptomatic anemia that necessitates red cell transfusions, making the *anemia of prematurity* unacceptable as a “purely physiologic” event.

Physiologic factors that influence erythropoiesis and the biologic characteristics of EPO are critical in the pathogenesis of the anemia of prematurity. Growth is extremely rapid during the first weeks of life, and red cell production by neonatal marrow must increase commensurately to avoid a decreasing hematocrit caused by an insufficient number of circulating red cells being diluted within the expanding blood volume. It is widely accepted that the

circulating life span of neonatal red cells in the bloodstream is shorter than that of adult red cells. This shorter survival of neonatal red cells, quite possibly, is an artifact, in part, because studies of transfused autologous red cells labeled with biotin or radioactive chromium may underestimate red cell survival in the infant's bloodstream for technical reasons. In healthy adults—when body size is stable so that blood and red cell volumes are constant (ie, not increasing with growing body size and commensurate increase in erythropoiesis), when no transfusions are given, and when large volumes of blood are not being taken for laboratory studies—the gradual disappearance of transfused labeled red cells, caused by dilution with red cells produced endogenously by the marrow, accurately reflects red cell survival in the bloodstream. In contrast, one or more of these confounding factors (ie, growth, red cell transfusions, phlebotomy) exists in infants—particularly, sick preterms—thus introducing error into the calculations performed when red cell survival is determined, based on disappearance of labeled red cells.

In addition, a key clinical factor is the need for repeated blood sampling to monitor the condition of critically ill neonates. Small preterm infants are the most critically ill, require the most frequent blood sampling, and suffer the greatest proportional loss of red cells because their circulating cell volumes are smallest. In the past, the mean volume of blood removed for sampling has been reported to range from 0.8 to 3.1 mL/kg/day during the first few weeks of life for preterm infants requiring intensive care. Promising “in-line” devices that withdraw blood, measure multiple analytes, and then reinfuse the sampled blood have been reported.<sup>3,4</sup> They have decreased the need for red cell transfusions. However, until these devices are proven more extensively to be practical, effective, and safe, replacement of blood losses from phlebotomy will remain a critical factor responsible for transfusions given to critically ill neonates—particularly, transfusions given during the first 4 weeks of life.

A key reason that the nadir hemoglobin values of preterm infants are lower than those of term infants is that preterm infants have a relatively diminished EPO plasma level in response to anemia.<sup>5</sup> Although anemia provokes EPO production in premature infants, the plasma levels achieved in anemic infants, at any given hematocrit, are lower than those observed in comparably anemic older persons.<sup>6</sup> Erythroid progenitor cells of preterm infants are quite responsive to EPO *in vitro*—a finding suggesting that inadequate production of EPO (not marrow unresponsiveness) is the major cause of physiologic anemia.<sup>7</sup>

The mechanisms responsible for the diminished EPO output by preterm neonates are only partially defined and, likely, are multiple. One mechanism is that the primary site of EPO production in preterm infants is in the liver, rather than kidneys.<sup>8</sup> This dependency on hepatic EPO is important because the liver is less sensitive to anemia and tissue hypoxia—resulting in a relatively sluggish EPO response to the decreasing hematocrit. The timing of the switch from the liver to kidneys is set at conception and is not accelerated by preterm birth. Viewed from a teleologic perspective, decreased hepatic production of EPO under in-utero

conditions of tissue hypoxia may be an advantage for the fetus. If this were not the case, normal levels of fetal hypoxia in utero could trigger high levels of EPO and produce erythrocytosis and consequent hyperviscosity. Following birth, however, diminished EPO responsiveness to tissue hypoxia is disadvantageous and leads to anemia because it impairs compensation for low hematocrit levels caused by rapid growth and red cell losses caused by phlebotomy, clinical bleeding, hemolysis, etc.

Diminished EPO production cannot entirely explain low plasma EPO levels in preterm infants, because extraordinarily high plasma levels of EPO have been reported in some fetuses and infants.<sup>9,10</sup> Moreover, macrophages from human cord blood produce normal quantities of EPO messenger RNA and protein.<sup>11</sup> Thus, additional mechanisms contribute to diminished EPO plasma levels. For example, plasma levels of EPO are influenced by metabolism (clearance) as well as by production. Data in human infants<sup>12</sup> have demonstrated low plasma EPO levels resulting from increased plasma clearance, increased volume of distribution, more rapid fractional elimination, and shorter mean plasma residence times than comparative values in adults. Thus, accelerated catabolism accentuates the problem of diminished EPO production, so that the low plasma EPO levels are a combined effect of decreased synthesis plus increased metabolism.

### Red Cell Transfusion Practices

Red cell transfusions are given to maintain the hematocrit at a level judged best for the clinical condition of the infant.<sup>13</sup> General guidelines acceptable to most neonatologists are listed in Table 30-1. However, many aspects of neonatal red cell transfusion therapy are controversial and vary from center to center. This lack of consistency stems from incomplete knowledge of the cellular and molecular biology of erythropoiesis during the perinatal period, of the pathophysiologic effects of neonatal anemia, and of the infant's response to red cell transfusions. In some instances the value of red cell transfusions is clear (eg, to manage anemia that has caused congestive heart failure), but in others it is not (eg, to correct irregular patterns of heart or respiratory rates), and practices are based largely on logical assumptions. Although well-designed clinical trials are being reported,<sup>14,15</sup> they are not completely mutually supportive and questions

**Table 30-1.** Guidelines for Small-Volume Red Cell Transfusions

- 
- Maintain >40% to 45% hematocrit for *severe* cardiopulmonary disease
  - Maintain >30% to 35% hematocrit for *moderate* cardiopulmonary disease
  - Maintain >30% to 35% hematocrit for *major* surgery
  - Maintain >20% to 25% hematocrit for infants with *stable* anemia, especially with:
    - Unexplained breathing disorders
    - Unexplained tachycardia
    - Unexplained poor growth
- 

Words in *italics* must be defined locally. For example, “severe” pulmonary disease may be defined as requiring mechanical ventilation with >0.35 FiO<sub>2</sub> and “moderate” as less intensive assisted ventilation.

remain. Therefore, it is important that pediatricians critically evaluate the guidelines in Table 30-1 and apply them in light of neonatal practice at their respective institutions.

An important controversy that is still unresolved is the wisdom—or lack, thereof—of prescribing Red Blood Cell (RBC) units for neonates using restrictive guidelines (ie, low pretransfusion hematocrit values) vs liberal guidelines (ie, conventional, relatively high pretransfusion hematocrit values). Two randomized controlled trials have been published<sup>14,15</sup> and, although many of their results agree, they disagree in one extremely important way—specifically, whether preterm infants are at increased risk of brain injury when given RBCs per restrictive guidelines (ie, due to undertransfusion). In both trials, preterm infants were randomly assigned to receive small-volume RBC transfusions per either restrictive or liberal guidelines—with guidelines based on a combination of the pretransfusion hematocrit or hemoglobin level, age of the neonate, and clinical condition at the time each transfusion was given. Both studies found that neonates in the restrictive transfusion group received fewer transfusions, without an increase in mortality or in morbidity based on several clinical outcomes. However, one critical discrepancy was present. Bell et al<sup>14</sup> found increases in apnea, intraventricular bleeding, and brain leukomalacia in infants transfused per restrictive guidelines, whereas Kirpalani et al<sup>15</sup> found no differences between infants in the restrictive vs liberal groups. Moreover, rates of serious outcomes were fairly high in both groups of the Kirpalani study—perhaps, a result of the extreme prematurity of the infants. However, because neonates in the liberal transfusion group of Bell et al likely had substantially higher hematocrit/hemoglobin levels than neonates in the liberal group of Kirpalani et al (an average hematocrit of 6% or hemoglobin level of 2 g/dL higher), it is reasonable to speculate that the higher hematocrit levels, in some way, protected liberally transfused infants in the Bell study.<sup>14,15</sup> Until more information is available, it seems wise to transfuse preterm neonates using conventional, relatively liberal guidelines (ie, do not place infants at possible risk of undertransfusion by strict conservative practices). The rationale for the conventional, relatively liberal guidelines (Table 30-1) follow in the paragraphs below.

#### **Maintain Hematocrit >40% to 45% for Severe Cardiopulmonary Disease**

In neonates with severe respiratory disease, such as those requiring high volumes of oxygen with ventilator support, it is customary to maintain the hematocrit above 40% to 45% (hemoglobin concentration above 13-14 g/dL)—particularly when blood is being drawn frequently for testing. This practice is based on the belief that transfused donor red cells, containing adult hemoglobin with its superior interaction with 2,3-diphosphoglycerate (2,3-DPG), will provide optimal oxygen delivery throughout the period of diminished pulmonary function. Consistent with this rationale for ensuring optimal oxygen delivery in neonates with pulmonary failure, it seems logical to maintain the hematocrit

above 40% in infants with congenital heart disease that is severe enough to cause either cyanosis or congestive heart failure.

#### **Maintain Hematocrit >30% to 35% for Moderate Cardiopulmonary Disease**

Following similar logic, it seems reasonable to maintain the hematocrit above 30% to 35% for moderate cardiopulmonary disease (Table 30-1).

#### **Maintain Hematocrit >30% for Major Surgery**

Definitive studies are not available to establish the optimal hematocrit for neonates facing major surgery. However, it seems reasonable to maintain the hematocrit >30% because of limited ability of the neonate's heart, lungs, and vasculature to compensate for anemia. Additional factors include the inferior off-loading of oxygen to tissues by the infant's own red cells because of the diminished interaction between fetal hemoglobin and 2,3-DPG plus the developmental impairment of neonatal pulmonary, renal, hepatic, and neurologic function. Because this transfusion guideline is simply a recommendation—not a firm indication—it should be applied with flexibility to individual infants facing surgical procedures of varying complexity (ie, minor surgery may be judged not to require a hematocrit >30%). The amount of anticipated blood loss must be strongly considered in preoperative transfusion decisions—with a likelihood of large blood loss, some physicians might like the preoperative hematocrit to be relatively high.

#### **Maintain Hematocrit >20% to 25% for Stable Infants with Symptomatic Anemia**

The clinical recommendations for red cell transfusions in preterm infants who are not critically ill but, nonetheless, develop moderate anemia (hematocrit <25% or hemoglobin level <8 g/dL) are extremely variable. In general, infants who are clinically stable with modest anemia do not require red cell transfusions, unless they exhibit significant clinical problems that are ascribed to the presence of anemia or are predicted to be corrected by donor red cells.<sup>16,17</sup> As an example, proponents of red cell transfusions to treat disturbances of cardiopulmonary rhythms believe that a low blood level of red cells contributes to tachypnea, dyspnea, apnea, and tachycardia or bradycardia because of decreased oxygen delivery to the respiratory center of the brain. Red cell transfusions might decrease the number of apneic spells by improving oxygen delivery to the central nervous system. However, results of clinical studies have been contradictory.<sup>14,16,17</sup>

Another controversial clinical indication for red cell transfusions is to maintain a reasonable hematocrit level as treatment for unexplained growth failure. Some neonatologists consider poor weight gain to be an indication for transfusion, particularly if the hematocrit is <25% and if other signs of distress are evident (eg, tachycardia, respiratory difficulty, weak suck and cry, and diminished activity).<sup>18</sup> In this setting, growth failure has been ascribed to the increase in metabolic expenditure required to support the work of labored breathing. In the past, a

hematocrit below 30% was of concern and often led to transfusion. However, results of clinical studies have not supported this practice,<sup>16,17</sup> and no apparent rationale exists to justify maintaining any predetermined hematocrit level by prophylactic, small-volume red cell transfusions in stable, growing infants who seem to be otherwise healthy.

In practice, the decision of whether to transfuse is based on the desire to maintain the hematocrit concentration at a level judged to be most beneficial for the infant's clinical condition. Investigators who believe this "clinical" approach is too imprecise have suggested the use of "physiologic" criteria such as red cell mass,<sup>19</sup> available oxygen,<sup>20</sup> mixed venous oxygen saturation, and measurements of oxygen delivery and utilization<sup>21</sup> to develop guidelines for transfusion decisions. In one study of 10 human infants with severe (oxygen-dependent) bronchopulmonary dysplasia, improvement of physiologic endpoints was shown (increased systemic oxygen transport and decreased oxygen use) as a consequence of small-volume red cell transfusions.<sup>21</sup> However, these promising but technically demanding methods are, at present, difficult to apply in the day-to-day practice of neonatology. The application, in infants, of data obtained from studies of animals and adult humans that correlate tissue oxygenation with the clinical effects of anemia and the need for red cell transfusions is confounded by the differences between infants and adults in hemoglobin oxygen affinity, ability to increase cardiac output, and regional patterns of blood flow.

Another physiologic factor considered in transfusion decisions is use of the circulating red cell volume/mass rather than the hematocrit or hemoglobin level. Although circulating red cell volume/mass is a potentially useful index of the blood's oxygen-carrying capacity, it cannot be predicted accurately from hematocrit or hemoglobin concentration levels; hence, it must be measured directly.<sup>22</sup> Unfortunately, circulating red cell volume/mass measurements and other "physiologic" criteria for transfusions are not widely available for clinical use.

### Red Blood Cell Products to Transfuse

Most transfusions are given to preterm infants as small-volume transfusions (10 to 20 mL/kg body weight) of RBCs in extended-storage media (additive solution AS-1, AS-3, AS-5) at a hematocrit of approximately 55% to 60% (Table 30-2). Alternatively, some neonatologists prefer RBCs in citrate-phosphate-dextrose-adenine (CPDA) solution at a hematocrit of approximately 70%—although the superiority of this last solution over extended-storage media has not been shown. Some centers prefer to centrifuge RBC aliquots before transfusion to prepare a uniformly packed RBC concentrate (hematocrit >80%).<sup>23</sup> Most RBC transfusions are infused slowly over 2 to 4 hours. Because of the small quantity of extracellular fluid (storage media) infused very slowly with small-volume transfusions, the type of anticoagulant-preservative solution used poses no risk for most premature infants.<sup>24,25</sup> Accordingly, the traditional use of relatively fresh RBCs (<7 days of storage) has been replaced in many centers by the practice of transfusing aliquots

**Table 30-2.** Formulation of Red Cell Anticoagulant-Preservative Solutions

| Constituent                       | CPDA | AS-1             | AS-3             | AS-5             |
|-----------------------------------|------|------------------|------------------|------------------|
| Volume (mL)                       | 63*  | 100 <sup>†</sup> | 100 <sup>†</sup> | 100 <sup>†</sup> |
| Sodium chloride (mg)              | None | 900              | 410              | 877              |
| Dextrose (mg)                     | 2010 | 2200             | 1100             | 900              |
| Adenine (mg)                      | 17.3 | 27               | 30               | 30               |
| Mannitol (mg)                     | None | 750              | None             | 525              |
| Trisodium citrate (mg)            | 1660 | None             | 588              | None             |
| Citric acid (mg)                  | 206  | None             | 42               | None             |
| Sodium phosphate (monobasic) (mg) | 140  | None             | 276              | None             |

\* Approximately 450 mL of donor blood is drawn into 63 mL of citrate-phosphate-dextrose-adenine (CPDA) solution. One Red Blood Cell unit (hematocrit, ~70%) is prepared by means of centrifugation and removal of most plasma. Results of calculations will be slightly different if 500 mL of donor blood is drawn.

<sup>†</sup> When additive solution AS-1 or AS-5 is used, 450 mL of donor blood is first drawn into 63 mL of CPD, which is identical to CPDA except it contains 1610 mg dextrose per 63 mL and has no adenine. When AS-3 is used, donor blood is drawn into CP2D, which is identical to CPD except it contains double the amount of dextrose. After centrifugation and removal of nearly all plasma, the cells are resuspended in 100 mL of the additive solution (AS-1, AS-3 or AS-5) at a hematocrit of approximately 55% to 60%.

of RBCs from a dedicated unit (or part of a unit) of RBCs stored up to 42 days in efforts to diminish the high donor exposure rates among infants who undergo numerous transfusions.<sup>23,25</sup> Neonatologists who object to prescribing stored RBCs and insist on transfusing fresh RBCs generally express the following four concerns: 1) the increase in the level of potassium in the plasma (ie, supernatant fluid); 2) the decrease in the level of 2,3-DPG; 3) the possible risks of additives such as mannitol and the relatively large amounts of glucose (dextrose) and phosphate present in extended-storage preservative solutions; and 4) the changes in red cell shape and deformability that may lead to poor flow through the microvasculature. Although these concerns often are legitimate for large-volume ( $\geq 25$  mL/kg) transfusions, particularly when infusion is rapid, they do not apply to small-volume transfusions for the following reasons.

After 42 days of storage in extended-storage media (AS-1, AS-3, AS-5) at a hematocrit of approximately 60%, extracellular ("plasma") potassium levels in RBC units approximate 50 mEq/L (0.05 mEq/mL), a value that at first glance seems alarmingly high. Simple calculations, however, show the actual dose of ionic potassium transfused in the extracellular "plasma" fluid is small. An infant weighing 1 kg given a 15-mL/kg transfusion of RBCs stored 42 days in extended-storage media at a hematocrit of 55% to 60% receives only 0.3 mEq. The potassium concentration of RBCs stored in CPDA solution at a hematocrit of 70% increases to 70 to 80 mEq/L after the 35 days of permitted storage, and the dose of potassium infused with a 15-mL/kg transfusion to a 1-kg infant is 0.3 to 0.4 mEq. These doses are quite small when compared to the usual daily potassium requirement of 2 to 3 mEq/kg and have been shown in several clinical studies not to cause hyperkalemia.<sup>25</sup>



**Table 30-3.** Quantity (Total mg/kg) of Additives Infused During a Transfusion of 15 mL/kg AS-1, AS-3, or AS-5 RBCs at Hematocrit 60%

| Additive        | AS-1  | AS-3 | AS-5  | Toxic Dose*    |
|-----------------|-------|------|-------|----------------|
| Sodium chloride | 54.0  | 24.6 | 52.6  | 137 mg/kg/day  |
| Dextrose        | 132.0 | 66.0 | 54.0  | 240 mg/kg/hour |
| Adenine         | 1.6   | 1.8  | 1.8   | 15 mg/kg/dose  |
| Citrate         | Trace | 37.8 | Trace | 180 mg/kg/hour |
| Phosphate       | Trace | 16.6 | Trace | >60 mg/kg/day  |
| Mannitol        | 45.0  | None | 31.5  | 360 mg/kg/day  |

\*The accuracy of toxic dose is difficult to predict because infusion rates are slow, allowing metabolism and distribution of additives from blood into extravascular sites. In addition, dextrose, adenine, and phosphate enter red cells and are somewhat sequestered and not immediately “available” in the extracellular solution. Data on potential toxic doses from Luban et al.<sup>24</sup>

Calculations for quantity of additives infused assumed the following: 1) total volume of RBC unit = 300 mL, consisting of 100 mL additive solution + 180 mL completely packed red cells + 20 mL of primary anticoagulant trapped between the red cells; 2) 15 mL/kg transfusion = 9 mL red cells + 6 mL additive solution; 3) quantity of additive solution infused per single RBC transfusion = quantity in 100 mL/(100 × 6); 4) trace amounts of trisodium citrate, citric acid, and phosphate were carried over by primary anticoagulant trapped between red cells.

By 21 days of storage, 2,3-DPG is totally depleted from RBCs as reflected by a  $P_{50}$  value that decreases from approximately 27 mm Hg in fresh blood to 18 mm Hg in stored red cells at the time of outdate. Because of the effects of high fetal hemoglobin levels in neonatal red cells, the 18 mm Hg value of red cells transfused after maximum storage corresponds to the expectedly low  $P_{50}$  value obtained from studies of red cells produced by many healthy preterm infants at birth. Thus, both older stored red cells and endogenous red cells from neonates have a similarly reduced ability to off-load oxygen compared with fresh adult red cells. However, older adult red cells in banked RBC units provide an advantage over the infant’s own cells because 2,3-DPG and the  $P_{50}$  of transfused adult cells (but not endogenous infant red cells) increase rapidly after transfusion. When studied in the setting of small-volume (15 mL/kg) transfusion, 2,3-DPG levels were maintained in infants given stored RBCs.<sup>26</sup>

The quantity of additives present in RBCs in extended-storage media is unlikely to be dangerous for neonates given small-volume transfusions (15 mL/kg).<sup>24,25</sup> Regardless of the type of suspending solution, the quantity of additives is quite small in the clinical setting in which a neonate would receive a single, small-volume transfusion over 2 to 4 hours. With AS-1, AS-3, and AS-5 as examples (Table 30-3), the dose of extended-storage media additives transfused during a typical small-volume transfusion is estimated to be far less than levels believed to be toxic.<sup>24</sup> This assumption was proved correct in clinical studies in which infants received one or more transfusions.<sup>24</sup> The safety and efficacy of transfusing stored RBCs is documented by the results of several laboratory tests performed before and after small-volume RBC transfusions in preterm infants (Table 30-4).

During storage in conventional preservative solutions, red cells sustain decreases in 2,3-DPG and adenosine triphosphate, and

**Table 30-4.** Mean Change in Blood Chemistry Levels During RBC Transfusions<sup>27,28</sup>

| Value             | Change*                          |                                   |
|-------------------|----------------------------------|-----------------------------------|
|                   | 1 to 21 Days of Storage (n = 78) | 22 to 42 Days of Storage (n = 42) |
| Hematocrit (%)    | + 12 ± 5                         | + 12 ± 4                          |
| Glucose (mg/dL)   | − 12 ± 24                        | − 16 ± 28                         |
| Lactate (mmol/L)  | − 0.6 ± 1.1                      | − 0.2 ± 0.3                       |
| pH                | 0.00 ± 0.08                      | 0.00 ± 0.06                       |
| Calcium (mg/dL)   | − 0.1 ± 0.5                      | 0.0 ± 0.8                         |
| Sodium (mEq/L)    | + 0.3 ± 4.6                      | − 0.4 ± 4.7                       |
| Potassium (mEq/L) | + 0.2 ± 0.8                      | + 0.2 ± 0.6                       |

\*Change (± SD) is posttransfusion value minus pretransfusion value. Statistical tests used were *t* test for pH, sodium, potassium, and glucose (normal distribution) and Wilcoxon rank sum test for hematocrit, calcium, and lactate (abnormal distribution). No statistically significant differences were found (p values all >0.05) comparing 1 to 21 days vs 22 to 42 days of storage.

they undergo membrane and cytoskeletal changes that lead to the formation of echinocytes and microvesicles and to decreased deformability. The last changes may lead to diminished perfusion of the microvasculature and, consequently, to tissue and organ dysfunction. For the past few years, the argument has raged over whether critically ill adult patients are harmed by receiving “old” RBC units (usually defined as stored ≥15 or ≥21 days), and whether mortality, multi-organ failure, infections, need for mechanical ventilation, length of stay in intensive care units and in the hospital, etc could be diminished by transfusing only fresh RBC units. Older, largely observational, studies generally support the idea that “old” RBC units put critically ill patients at risk, particularly if they receive multiple transfusions. Several more recent studies have challenged this concept and have cautioned against insisting on transfusions of fresh RBCs.<sup>29,30</sup> Well-designed clinical trials are needed to resolve this question.

The situation is similar for neonatal RBC transfusions (ie, neonates are often critically ill and questions about morbidity caused by transfusing stored RBCs have been raised). However, well-designed trials have addressed efficacy and safety and, within the limits of the number of infants studied, fresh and stored cells have been documented to be equivalent.<sup>14,25,26</sup> Moreover, the intravascular recovery 24 hours after transfusion and long-term survival of stored red cells is normal, when measured in human infants using biotinylated red cells.<sup>31</sup> Because the risks of multiple donor exposure can be nearly eliminated by giving infants cells taken from dedicated, stored RBC units and because increased risks of transfusing stored vs fresh cells have not been demonstrated, it seems prudent to continue transfusing stored RBCs for small-volume transfusions.

**Approach to Anemia of Prematurity**

For many infants with a birthweight less than 1.0 kg, anemia of prematurity is severe and necessitates therapy with red cell

transfusions and/or the administration of recombinant human erythropoietin (rHuEPO) plus iron.

For decades, red cell transfusion has been the standard of care and continues to be so. A key principle is that allogeneic donor exposure should be minimized by transfusing cells from dedicated RBC units stored as long as permitted (eg, 42 days for extended-storage anticoagulant-preservative solutions). At the University of Iowa DeGowin Blood Center, preterm infants who need transfusions are assigned to dedicated RBC units suspended in extended-storage (42-day) solutions. At the time the first transfusion is ordered, one-half of a freshly collected unit (stored  $\leq 7$  days) is dedicated to a preterm infant with a birthweight of 1.0 kg or less. The rest of the unit can be assigned to another infant. Thus, one complete unit can serve two very-low-birthweight infants simultaneously. When transfusions are ordered, aliquots are removed sterily and issued.<sup>23</sup> Although units are used throughout 42 days of storage, if a unit has been stored 14 days without an infant being assigned to it, it has become relatively aged (14 of its 42 storage days have lapsed), and it enters the general inventory to be used for older patients as needed. This plan has been demonstrated to be cost-effective.<sup>32</sup>

Recognition of low plasma EPO levels and adequate erythropoietic activity in preterm infants provides a rational basis to consider rHuEPO as treatment for the anemia of prematurity. Because the inadequate quantity of EPO is the major cause of anemia—not a subnormal response of erythroid progenitors to EPO—it is logical to assume that rHuEPO will correct EPO deficiency and will effectively treat the anemia of prematurity. Unfortunately, rHuEPO has not been widely accepted in clinical neonatology practice because its efficacy is incomplete. On one hand, clonogenic erythroid progenitors from neonates respond well to rHuEPO in vitro and rHuEPO and iron effectively stimulate erythropoiesis in vivo as evidenced by increased blood reticulocyte and red cell counts in recipient infants (ie, efficacy successful at the marrow level). On the other hand, when the primary goal of rHuEPO therapy is to eliminate transfusions, rHuEPO often fails (ie, efficacy at the clinical level has not been consistently successful).<sup>13,33</sup>

By 2000, over 20 controlled clinical trials assessing the efficacy of rHuEPO to eliminate RBC transfusions in the anemia of prematurity were published with inconsistent results. To investigate the extent and reasons for the inconsistencies, a meta-analysis was conducted of the controlled clinical studies published between 1990 and 1999.<sup>33</sup> To be included, a reported study had to prospectively enroll a treatment group of preterm infants under 4 months of age treated with rHuEPO and a concurrent control group not given rHuEPO. Twenty-one reports were eligible for inclusion in the meta-analysis. However, because the experimental design and conduct of the studies were extremely variable, only four reports were judged to fulfill all of the highly desired characteristics of being effectively blinded, having high-quality experimental design (ie, randomized, placebo-controlled, all dropouts well-explained, etc), using conservative transfusion practices (rather than liberal transfusions, which suppress

endogenous erythropoiesis), and enrolling a majority of very preterm infants with birthweights  $< 1.0$  kg.

Two major conclusions emerged from the meta-analysis.<sup>33</sup> First, the controlled trials of rHuEPO to treat the anemia of prematurity differed from one another in multiple ways and, consequently, produced markedly variable results that could not be adequately explained. Hence, it was judged impossible to make firm recommendations regarding use of rHuEPO in clinical practice to treat the anemia of prematurity. Second, when the four studies with highly desired characteristics were analyzed separately, rHuEPO was found to be efficacious in significantly reducing transfusion needs. However, the magnitude of the effect of rHuEPO on reducing the total red cells transfusions given to infants throughout their initial hospitalization was, in fact, relatively modest and of questionable clinical importance. Although the meta-analysis was unable to recommend how to use rHuEPO in clinical practice, it was apparent that: 1) relatively large or stable preterm infants, shown to respond best to rHuEPO plus iron at the marrow level, are given relatively few red cell transfusions with today's conservative transfusion practices and, accordingly, have little need for rHuEPO when the goal is to avoid transfusions; and 2) extremely small preterm infants, who are critically ill and unstable, have not consistently responded to rHuEPO plus iron when the outcome measure is to reduce need for transfusions.

Several reports published after 2000 have provided useful information. Donato et al,<sup>34</sup> assessing the effects of rHuEPO very early in life, randomly assigned 114 neonates with birthweights  $< 1.25$  kg to receive either rHuEPO or placebo during the first 2 weeks of life, followed by a 6-week treatment period during which *all infants* were given rHuEPO, iron, and folic acid. During the first 3 weeks of life, rHuEPO increased reticulocytes and hematocrit values, but there was no difference in red cell transfusions. However, at the end of all treatment (8 weeks), a subgroup of infants with birthweights  $< 0.8$  kg and phlebotomy losses  $> 30$  mL/kg who were given rHuEPO shortly after birth received fewer total red cell transfusions than infants initially given placebo ( $3.4 \pm 1.1$  vs  $5.4 \pm 3.7$ ,  $p < 0.05$ ). Similarly, Yeo et al<sup>35</sup> found a modest advantage for a subgroup of very-low-birthweight infants given rHuEPO. Infants with birthweights 0.8 to 0.99 kg were given fewer transfusions with rHuEPO than control infants not given rHuEPO ( $2.1 \pm 1.9$  vs  $3.5 \pm 1.6$ ,  $p < 0.04$ ). A randomized blinded trial by Meyer et al<sup>36</sup> found an advantage for very-low-birthweight infants given rHuEPO. Neonates with birthweights  $< 1.7$  kg plus criteria that predicted a likely need for red cell transfusions were randomly assigned either to receive rHuEPO beginning shortly after birth or to experience a sham treatment to simulate placebo injections. Iron was given to all infants, but at a much lower dose (unfortunately) to control infants not given rHuEPO than to infants given rHuEPO—thus, creating two variables/differences (rHuEPO and iron dose, rather than just rHuEPO) being assessed in treated vs control infants. There was no overall difference in red cell transfusions, but in a subset of infants with birthweights  $< 1.0$  kg, transfusions given

late (ie, after 30 days of age) were reduced by rHuEPO vs transfusions given to controls ( $0.5 \pm 0.7$  vs  $1.6 \pm 1.1$ ,  $p = 0.01$ ).

Two reports defined rHuEPO “success” as maintaining a hematocrit of  $\geq 30\%$  without need for any transfusions. Maier et al<sup>37</sup> randomly assigned 219 neonates with birthweights 0.5 to 0.99 kg to receive early rHuEPO (from the first week of life for 9 weeks), late rHuEPO (from the fourth week of life for 6 weeks), or no rHuEPO. “Success” was modest (13% with early rHuEPO, 11% with late rHuEPO, and 4% with no rHuEPO). Only early rHuEPO administration was significantly superior to no rHuEPO ( $p = 0.026$ ). Avent et al<sup>38</sup> randomly assigned 93 neonates with birthweights 0.9 to 1.5 kg to receive low-dose rHuEPO (250 units/kg), high dose rHuEPO (400 units/kg), or no rHuEPO. Treatment began within 7 days of life and continued until discharge (median 32 days and maximum 74 days). “Success” was met by 75% of low-dose rHuEPO infants, 71% of high-dose rHuEPO infants, and 40% of no rHuEPO infants ( $p < 0.001$ ). The number of red cell transfusions given to all infants treated with rHuEPO vs those with no rHuEPO was not significantly different. The authors concluded that rHuEPO does not significantly further decrease red cell transfusions in infants with birthweights of 0.9 to 1.5 kg, when phlebotomy losses are small and relatively few transfusions are given per stringent transfusion guidelines.

The observation by Avent et al<sup>38</sup> that the benefits of rHuEPO in reducing the number of red cell transfusions given can be equalled by stringent or conservative transfusion guidelines has been made by others. Franz and Pohlandt<sup>39</sup> assessed both the number of red cell transfusions given and red cell transfusion guidelines in four prospective, randomized trials of rHuEPO given to preterm neonates. To be selected for analysis, the clinical trials had to include ventilated infants (ie, sick infants likely to receive red cell transfusions). The authors found that, when restrictive transfusion guidelines were followed, the number of red cell transfusions and the volume of red cells transfused were similarly low in infants either given or not given rHuEPO.<sup>36</sup> Similarly, Amin and Alzahrani<sup>40</sup> found no difference in the number of red cell transfusions, whether or not rHuEPO was given to preterm infants with birthweights  $\leq 1.0$  kg, when transfusions were given per strict transfusion guidelines.

Another alternative to allogeneic transfusions is use of placental blood as a source of autologous red cells.<sup>41,42</sup> Although collection, storage, and transfusion of placental blood after delivery deserves careful consideration, at least three obstacles must be overcome before autologous transfusions of stored placental red cells can be adopted for clinical use. One concern is that a sufficient quantity of red cells will not be obtained consistently from placentas of preterm infants to avoid a significant number of allogeneic transfusions. The second issue is the sterility of placental blood. Because bacterial contamination of placental blood is a distinct possibility,<sup>41</sup> extensive testing will be needed to ensure absolute safety. Finally, acceptable quality of stored placental blood must be maintained.<sup>42</sup> Because of these concerns, collection and storage of placental blood has not been encouraged.<sup>43</sup>

Instead, interest has been renewed in delayed clamping of the umbilical cord immediately following delivery to expand neonatal blood volume and red cell volume/mass to, possibly, improve circulatory hemodynamics and increase tissue perfusion and oxygenation.<sup>22,44</sup>

## Platelet Transfusion

### Pathophysiology of Neonatal Thrombocytopenia

Blood platelet counts of  $\geq 150,000/\mu\text{L}$  are present in normal fetuses (as early as 17 weeks of gestation) and in normal neonates. Thus, platelet counts are in the normal range, regardless of gestational age at birth. Low platelet counts indicate potential problems. Preterm neonates commonly have thrombocytopenia (eg, in one neonatal intensive care unit, 22% of infants had platelet counts  $< 150,000/\mu\text{L}$ ).<sup>45</sup> In contrast, thrombocytopenia is documented in  $< 1\%$  of term neonates.<sup>46</sup> Blood platelet counts  $< 100,000/\mu\text{L}$  pose significant clinical risks. In one study, neonates with birthweights less than 1.5 kg and a platelet count  $< 100,000/\mu\text{L}$  were compared with neonates of similar size who did not have thrombocytopenia.<sup>47</sup> The bleeding time was prolonged when platelet counts were  $< 100,000/\mu\text{L}$ , and platelet dysfunction was suggested by bleeding times that were disproportionately long for the degree of thrombocytopenia present. The incidence of intracranial hemorrhage was 78% among thrombocytopenic neonates with a birthweight less than 1.5 kg vs 48% for similar neonates without thrombocytopenia. In addition, the extent of hemorrhage and neurologic morbidity was greater among infants with thrombocytopenia.<sup>47</sup>

Although multiple pathogenetic mechanisms underlying thrombocytopenia likely are involved in these sick neonates, accelerated platelet destruction frequently is implicated by shortened platelet survival time, increased level of platelet-associated immunoglobulin G, increased platelet volume, normal number of marrow megakaryocytes, and an inadequate response to platelet transfusions.<sup>45,48</sup> Another mechanism that contributes to neonatal thrombocytopenia is diminished platelet production. This is evidenced by neonatal megakaryocytes that are smaller and of lower ploidy than those of adults.<sup>49</sup> In addition, some investigators have reported decreased numbers of megakaryocyte progenitors,<sup>50</sup> while others have not.<sup>49</sup> The response to thrombocytopenia in adults is to increase megakaryocyte number, volume, and ploidy—mediated primarily by thrombopoietin—all of which leads to increased platelet production. Thrombocytopenic neonates either fail to increase, or increase inadequately, megakaryocyte number, volume, and ploidy when compared to adults and, consequently, they cannot increase platelet counts sufficiently to correct thrombocytopenia.<sup>49,51</sup> Moreover, thrombopoietin seems to increase neonatal megakaryocyte proliferation, but inhibit endoreduplication,<sup>52</sup> in contrast, both are increased in adults.

As is reminiscent of neonatal red cell and neutrophil production, where basal erythropoiesis and myelopoiesis are adequate,

basal platelet production and thrombopoiesis are adequate. However, under the stress of cytopenia, none of the three cell lines is capable of increasing production to completely and promptly correct low blood counts (ie, the infants lack sufficient hematopoietic reserve).

### Recommendations for Platelet Transfusion during Infancy

The relative risks of different degrees of thrombocytopenia in various clinical settings during infancy remain largely unanswered. Prophylactic platelet transfusions to prevent bleeding in preterm neonates were studied systematically several years ago, and the results still seem relevant.<sup>53</sup> No randomized clinical trials of therapeutic platelet transfusions have been reported for bleeding thrombocytopenic neonates. Recognizing the need for more data, it seems logical in the interim to transfuse thrombocytopenic neonates per the guidelines presented in Table 30-5.

Two firm indications for platelet transfusions are either to control hemorrhage that has already occurred or to prevent it from complicating an invasive procedure. No disagreement exists over using a pretransfusion platelet count of 50,000/ $\mu$ L as a minimum transfusion trigger in these instances. However, platelet transfusions are given at platelet counts  $>50,000/\mu$ L by some physicians to neonates to control bleeding or in hopes of reducing either the threat of, or the worsening of, intracranial hemorrhage in high-risk preterm neonates.<sup>47</sup> No data exist to clearly establish the efficacy of platelet transfusions at these relatively high platelet levels.

Conventional prophylactic platelet transfusions are given to prevent bleeding when severe thrombocytopenia poses a likely risk of spontaneous hemorrhage. Prophylactic platelet transfusions are given by some physicians to maintain the presence of a normal platelet count in hopes of preventing the neonate from slipping into high-risk situations that might lead to spontaneous bleeding. Regarding the first circumstance, most experts agree that it is reasonable to give platelets to any infant with a platelet count,  $<20,000/\mu$ L because spontaneous hemorrhage is a likely risk at this platelet count. Severe thrombocytopenia occurs most commonly among sick infants who, because of the illness, often receive medications that can compromise the function of their already diminished number of platelets. Because these factors are more pronounced in extremely preterm infants, who are clinically unstable, some neonatologists favor prophylactic platelet transfusion whenever the platelet count decreases to  $<50,000/\mu$ L, or even to  $<100,000/\mu$ L, in critically ill infants.<sup>47</sup>

**Table 30-5.** Guidelines for Neonatal Platelet Transfusions

- Maintain  $>50,000$  to  $100,000/\mu$ L platelets for *significant* bleeding
- Maintain  $>50,000/\mu$ L platelets for *invasive* procedures
- Maintain  $>20,000/\mu$ L platelets prophylactically for *clinically stable* neonates
- Maintain  $>50,000$  to  $100,000/\mu$ L platelets prophylactically for *clinically unstable* neonates

Words in italics must be defined locally. For example, consider bleeding site, extent and degree of prematurity, and underlying medical condition.

It is not efficacious to maintain a completely normal platelet count ( $\geq 150,000/\mu$ L) in preterm neonates without bleeding.<sup>53</sup> Moreover, such a practice can place infants at increased risk from more platelet donor exposures. Intracranial hemorrhage occurs commonly among sick preterm infants. Although neither a causative role for thrombocytopenia nor a therapeutic benefit for platelet transfusion has been established in this disorder, it seems logical to presume thrombocytopenia might be a risk factor.<sup>54</sup> Accordingly, in a randomized trial designed to address this issue, transfusion of platelets whenever the platelet count decreased below the normal value of  $150,000/\mu$ L (which maintained the average daily platelet count  $>200,000/\mu$ L) was compared with transfusion of platelets only when the platelet count decreased below  $50,000/\mu$ L.<sup>53</sup> There was no difference in the incidence of intracranial hemorrhage (28% vs 26%) in the two groups. Thus, there is no documented benefit to transfusing “prophylactic platelets” to maintain a completely normal platelet count in preterm neonates vs transfusing “therapeutic platelets” to treat thrombocytopenia when it actually occurs.

### Platelet Product to Transfuse

The ideal goal of platelet transfusions for many thrombocytopenic neonates is to increase the low pretransfusion platelet count to a posttransfusion count  $>50,000/\mu$ L and for sick preterm infants to  $>100,000/\mu$ L. This can be achieved consistently by transfusing 5 to 10 mL/kg of *unmodified* platelet concentrates (withdrawn from a unit of platelets collected either by centrifugation of fresh units of whole blood or plateletpheresis and transfused directly). Platelet concentrates should be transfused as rapidly as the neonate’s condition allows, certainly within 2 hours.

Routinely reducing the volume of platelet concentrates for infants by means of additional centrifugation steps is both unnecessary and unwise—unless a specific reason exists to do so. To illustrate, transfusion of 10 mL/kg platelet concentrate, taken directly from the unit and transfused, provides approximately 10,000/ $\mu$ L platelets. If the blood volume of an infant is 70 mL/kg body weight and the plasma volume is 40 mL/kg, the platelet dose of 10 mL/kg increases the platelet count 100,000 to 150,000/ $\mu$ L above the pretransfusion baseline—assuming a posttransfusion platelet recovery of 60%. This calculated increment is consistent with the increment actually achieved after transfusing this dose as reported in clinical studies.<sup>53</sup> With modest thrombocytopenia, a 5 mL/kg dose may be sufficient. In general, 5 to 10 mL/kg is not an excessive transfusion volume even for sick neonates, as long as the intake of other intravenous fluids, medications, and nutrients is adjusted. If volume reduction is to be achieved, the method of reduction *and* the efficacy of platelet transfusions after reduction must be validated *locally* to ensure the quantity and quality of platelets (both *ex vivo* and *in vivo*) remaining after modification.

In the selection of platelet units for transfusion, it is desirable for the infant and the platelet donor to be of the same ABO blood group. It is important to minimize repeated transfusions of group O platelets to group A or B recipients, because large quantities of

passive anti-A or anti-B can lead to hemolysis. This should be avoided easily, with the exception of a directed-donor situation in which the infant receives—usually after an emotional rather than medical decision—platelet transfusions from an out-of-group donor. Directed-donor platelets should not be used in this situation; ABO-identical platelets from the general inventory should be selected. Proven methods exist to reduce the volume of platelet concentrates when truly warranted (eg, multiple transfusions anticipated in which several doses of passive anti-A or anti-B may lead to hemolysis, or failure to respond to a transfusion of 10 mL/kg of unmodified platelet concentrate). However, additional processing should be performed with great care—using a method that is well-validated *locally*—because of probable platelet loss, clumping, and dysfunction caused by the additional handling.

Approach to Infants with Thrombocytopenia

Thrombocytopenia exists wherever the blood platelet count is <150,000/μL. Every infant with thrombocytopenia needs an evaluation—if nothing more than a repeated complete blood count, a review of the medical history, and a physical examination. Definitive management of thrombocytopenia depends on the underlying disorder and is beyond the scope of this discussion (see Chapter 23). Correction of the thrombocytopenia per se by means of platelet transfusions is based on maintaining a platelet count deemed appropriate for the infant’s clinical condition (Table 30-5).

There are no alternatives to platelet transfusion in the care of neonates with thrombocytopenia. Recombinant thrombopoietin (c-Mpl ligand or megakaryocyte growth and differentiation factor) and interleukin-11 are promising agents in older patients. However, neither is recommended for use during infancy, and both have potential toxicities that can preclude their use in the care of sick preterm infants. Thrombopoietin has broad actions on the early precursors of all three major lineages in the marrow and may produce effects in excess of those expected on megakaryocytes and platelets. Moreover, its interactions with fetal/neonatal megakaryocytes may be quite different from those with adults.<sup>52</sup> Interleukin-11 may cause anemia. Clearly, these agents must not be prescribed in the treatment of infants, except in experimental settings with parental consent and institutional oversight. As noted in the plasma transfusion section, recombinant activated Factor VII has been used “off-label” to treat life-threatening bleeding during infancy.

Plasma Transfusion

Pathophysiology of Neonatal Clotting Proteins

Hemostasis in a neonate is quantitatively and qualitatively different from that in an older child or adult, and the risk exists of either serious hemorrhage or thrombosis. Thus, some important facts about neonatal clotting proteins are summarized before issues of plasma transfusion are addressed. (See Chapters 17, 20, and 28 for more detailed discussions of hemostasis.)

Table 30-6. Guidelines for Neonatal Plasma Transfusions

- High risk of bleeding because of acquired deficiency of clotting proteins
  - Exchange transfusion
  - Extracorporeal membrane oxygenation
  - Cardiac bypass surgery
  - Disseminated (consumptive) coagulation
- Bleeding because of congenital clotting protein deficiency, if specific concentrate is unavailable
- Bleeding because of vitamin K deficiency
- Thrombosis because of anticoagulant protein deficiency

Dose depends on severity of the deficiency, but a satisfactory starting dose is 10 to 15 mL/kg body weight of Fresh Frozen Plasma or Plasma Frozen within 24 Hours After Phlebotomy.

Maternal clotting factors do not cross the placenta. Therefore, fetal levels depend on endogenous production. Clotting proteins are synthesized by the fetus beginning in the first trimester, with concentrations gradually increasing throughout gestation.<sup>55</sup> At birth, the mean levels of the contact factors (Factors XII and XI, prekallikrein, and high-molecular-weight kininogen) are approximately 40% to 50% of adult values in term neonates and approximately 30% to 40% of adult values in preterm neonates. The vitamin-K-dependent factors (II, VII, IX, and X) are present at approximately 40% to 50% of adult values in term neonates and at approximately 20% to 50% of adult values in preterm neonates. Strikingly low levels are present in very immature neonates. Neonatal levels of Factors VIII and XIII and fibrinogen are comparable with adult levels, although the level of Factor XIII can be quite low in some infants at birth. The natural anticoagulant proteins (antithrombin and proteins C and S) are 30% to 50% of adult values. Fibrinolysis is less well studied but probably is diminished to a considerable degree because the plasminogen level is moderately low.

Plasma Transfusion Practices

Clotting times are prolonged in neonates compared with results of older children and adults because of developmental deficiency of clotting proteins. Plasma should be transfused only after reference to normal values that take into account the birthweight and age of the infant. Guidelines for neonatal transfusion of plasma are presented in Table 30-6. Fresh Frozen Plasma (FFP) and Plasma Frozen within 24 Hours after Phlebotomy (PF24), whether prepared from whole blood units or by plasmapheresis, are used interchangeably. PF24 has up to a 25% loss of Factor VIII and less loss of Factor V; neither has clinical importance for the vast majority of patients receiving plasma transfusions.

The indications for plasma transfusion include reconstitution of RBC concentrates to simulate whole blood for use in massive transfusions (eg, exchange transfusion, extracorporeal membrane oxygenation, or cardiovascular surgery), provision of multiple clotting factors for bleeding caused by disseminated intravascular coagulation or vitamin K deficiency, and treatment of congenital factor deficiencies when more specific treatment

(purified and virus-inactivated factor concentrates) is not available or the diagnosis of a specific factor deficiency has not been made (ie, multiple factors may need replacement).

The use of prophylactic plasma transfusions to prevent intraventricular hemorrhage in preterm neonates is not recommended. Use of plasma as a suspending agent to adjust the hematocrit of RBC concentrates before small-volume transfusions (15 mL/kg) should be discouraged because plasma offers no medical benefit over simply transfusing red cells suspended in the preservative solution in which they are stored. Similarly, the use of plasma in partial exchange transfusion for the management of neonatal hyperviscosity syndrome (erythrocytosis) is unnecessary, because safer colloid solutions (eg, 5% albumin) are available.

In the treatment of bleeding neonates, cryoprecipitate often is considered as an alternative to plasma because of its small volume. However, cryoprecipitate contains only fibrinogen, von Willebrand factor, and Factors VIII and XIII. It is not effective for managing the more extensive multi-factor clotting deficiencies that are commonly encountered—despite the desirability of a small infusion volume. Discussion of the transfusion of clotting factor concentrates is presented in detail in Chapter 28. However, it is important to point out that recombinant activated Factor VII has been effective in treating life-threatening bleeding in infants with deficiency and/or dysfunction of clotting proteins or platelets.<sup>56</sup>

## Neutrophil Transfusion

### Pathophysiology of Neonatal Neutropenia

Neonates are unusually susceptible to severe bacterial infections, and multiple abnormalities of neonatal body defenses contribute to their pathogenesis. Neonatal neutrophils have both quantitative and qualitative abnormalities related to the increased incidence, morbidity, and mortality of bacterial infections. These abnormalities include absolute and relative neutropenia, diminished chemotaxis, abnormal adhesion and aggregation, defective cellular orientation and receptor capping, decreased deformability, inability to alter membrane potential during stimulation, imbalances of oxidative metabolism, and a diminished ability to withstand oxidant stress.<sup>57</sup>

Neutropenia can occur during fulminant bacterial infection. Because physiologic neutrophilia occurs soon after birth, it is quite unusual for the absolute blood neutrophil count to decrease to less than 2000/ $\mu$ L during the first week of life. Although an abnormally low neutrophil count can occur in neonates with disorders as diverse as sepsis, asphyxia, and maternal hypertension, suspicion of severe bacterial infection must always be high whenever relative neutropenia (neutrophil count  $<2000/\mu$ L) occurs in a sick neonate. The mechanisms responsible for abnormal neonatal granulopoiesis are only partially defined. One factor is that the postmitotic marrow neutrophil storage pool (metamyelocytes and mature, segmented neutrophils) is inadequate during fulminant infection. The neutrophil storage pool accounts for 26% to 60% of all nucleated cells in the marrow of normal

neonates, whereas neonates with sepsis may have a storage pool numbering less than 10% of nucleated marrow cells. Thus, they have severely diminished marrow neutrophil reserves.<sup>58</sup> Second, storage pool neutrophils are released at an excessively rapid and apparently poorly regulated rate from the marrow during stress. Third, committed (clonogenic) neutrophil precursors in neonatal marrow are fewer in number in neonates than in older patients, and most of these cells are actively proliferating even when studied at an apparently basal state.<sup>58,59</sup> Thus, neonatal marrow functions at near capacity in basal state and is unable to either rapidly expand production or release stored neutrophils to meet the increased demands of infection. Additional discussion of fetal and neonatal hematopoiesis is presented in Chapter 25.

### Neutrophil Transfusion Practices

Because both quantitative and qualitative abnormalities of neonatal neutrophils occur, neutrophil transfusions have been reported to successfully treat neonatal sepsis.<sup>60</sup> Neutrophil transfusions generally were given to neonates with fulminant sepsis and relative neutropenia (neutrophil count  $<2000$  to  $3000/\mu$ L during the first week of life or  $<1000/\mu$ L thereafter). Unfortunately, neutrophil transfusions have not provided a satisfactory solution to neonatal sepsis, and they are used rarely, if at all, for several reasons.<sup>60</sup> Although neutrophil transfusion is efficacious for some infants with neutropenia and fulminant sepsis, only neutrophil concentrates obtained by means of automated leukapheresis for transfusion have demonstrated effectiveness. The controlled trials contain scientific flaws, and in many instances, conventional supportive care with antibiotics seemed equally efficacious.

### Approach to Infants with Sepsis and Neutropenia

Each institution must assess its own experience with neonatal sepsis. If nearly all infants survive without apparent long-term morbidity when treated only with antibiotics, neutrophil transfusions are unnecessary, and attention should be focused on prompt diagnosis and optimal antibiotic therapy. If the outcome of standard therapy is not optimal, alternative therapies must be considered to improve the outlook. Such therapies include intravenous immunoglobulin (IVIG) and/or myeloid cytokines such as granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Most studies evaluating prophylactic IVIG to prevent neonatal infections have shown either little or only modest benefit, whereas several therapeutic studies have shown a benefit to adding IVIG to antibiotics to treat neonatal infections.<sup>61</sup> Data are insufficient to justify the use of IVIG as a standard of care for all preterm neonates to prevent or manage sepsis. However, it seems reasonable to give “physiologic” doses of IVIG (0.3 to 0.4 g/kg) to septic very-low-birthweight neonates because they are likely to have hypogammaglobulinemia as a result of extremely premature birth (ie, being born before major placental transport of immunoglobulin G has taken place). To date, properly designed clinical studies of recombinant myeloid growth factors given to

human neonates are limited,<sup>62,63</sup> and firm recommendations cannot be made for use of these agents in the management of neonatal neutropenia or sepsis.

There is no universally accepted role for neutrophil transfusions, IVIG, or myeloid growth factors as a standard of practice to treat neonatal sepsis. Until more information becomes available, it seems reasonable to manage fulminant sepsis in neonates with neutropenia (neutrophil counts  $<2000/\mu\text{L}$  during the first week of life or  $<1000/\mu\text{L}$  thereafter) as follows. For infants born before 30 weeks of gestation, give one dose of 0.4 g/kg of IVIG to correct possible hypogammaglobulinemia plus 5  $\mu\text{g/kg}$  of G-CSF or 10  $\mu\text{g/kg}$  GM-CSF on 3 consecutive days. For infants born at 30 weeks of gestation or after, give 5  $\mu\text{g/kg}$  of G-CSF or 10  $\mu\text{g/kg}$  GM-CSF on 3 consecutive days. This therapy should be adjunctive to optimal antibiotic and supportive care, and it must be given with parental consent and the understanding that its efficacy and potential toxicity are not completely understood. Actually, because of high plasma levels of endogenous G-CSF already present in some septic neonates, it has been cautioned that adding additional exogenous recombinant G-CSF offers no benefit.<sup>64</sup>

## Acknowledgment

This work was supported by National Institutes of Health grants P01 HL46925 and RR00059.

## Disclaimer

The author has disclosed no conflicts of interest.

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## 31

## Transfusion Support for the Oncology Patient

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Transfusion support for patients with cancer is a complicated, multifaceted medical therapy meant to overcome complications related to chemotherapy, radiation, transplantation, or widespread metastatic disease. Oncology patients are often substantially immunosuppressed and may require chronic transfusion support. For these patients, greater attention must be paid to the preparation, modification, and response to blood components to help ensure better outcomes. Furthermore, with recent evidence that drugs such as erythropoietin may be particularly harmful to oncology patients, heavier reliance on allogeneic blood transfusion may be necessary in the immediate future. This chapter highlights the most challenging aspects of transfusion therapy for oncology patients encountered by clinicians and transfusion services on a routine basis.

## Red Cell Transfusion

### Indications

Red cell transfusions should be used to increase the oxygen-carrying capacity of whole blood for oncology patients with anemia. Unfortunately, there are no oncology-specific evidence-based target laboratory criteria for the transfusion of Red Blood Cell (RBC) units. Therefore, oncology patients should be transfused on a symptomatic basis or at predetermined hemoglobin or hematocrit measurements as defined by oncology services. When clinicians apply standards used in other critically ill populations, and consider the underlying comorbid diseases, most oncology patients should tolerate hemoglobin levels of 7 to 10 g/dL without the need for allogeneic transfusions.<sup>1</sup> There is no evidence to show that sustained increases in hemoglobin over 10 g/dL provide any therapeutic benefit for ill patients; in fact, “hypertransfusion” strategies may actually be harmful to patient outcome.<sup>2</sup>

### Selection of ABO Group for RBC Transfusion

For the majority of oncology patients, no specific consideration is given to selection of ABO group of RBC units so long as the unit is ABO compatible with the recipient. However, for patients who have undergone allogeneic hematopoietic stem cell transplantation (HSCT), the choice of ABO group can be difficult, particularly if there is an ABO mismatch between hematopoietic progenitor cell (HPC) donor and recipient. ABO incompatibility, although not a barrier to HSCT, does complicate posttransplant management. Patients receiving ABO-mismatched transplants are known to require more transfusions because of delayed cellular engraftment and red cell aplasia. These patients are also at risk for acute and delayed red cell hemolysis caused by ABO incompatibility during HPC reinfusion and as engraftment takes place.<sup>3-7</sup> Despite these complications, numerous studies have shown that ABO-mismatched HSCT does not adversely affect long-term survival or outcome in either pediatric or adult populations.<sup>3,8,9</sup>

Although ABO mismatch does not seem to have long-term drawbacks, providing appropriate blood component support immediately following transplantation can be a complex endeavor for a transfusion service. In the setting of allogeneic transplantation, there are several possibilities for ABO antibody mismatch. Patients may have a minor mismatch, wherein the donor possesses antibodies against recipient cells. This would occur when a patient with group A red cells receives HPCs from a group O donor. Another possibility is a major mismatch, wherein a recipient harbors antibodies directed against donor cells. This would occur when a patient with group O red cells receives HPCs from a group A donor. For minor incompatibility, a strategy to reduce hemolysis during HPC infusion is plasma reduction to remove, or significantly lower, titers of offending antibodies.<sup>10</sup> Similarly, for major mismatches, techniques for red cell depletion can prevent immediate hemolysis during infusion.<sup>11</sup>

After transplantation, the selection of blood components can be difficult, because antibodies can persist for weeks or months after stem cell rescue.<sup>12,13</sup> Additionally, following HSCT,

patients may present as chimeras with two distinct blood group populations seen on routine type and screening tests.<sup>12</sup> Thus, strategies have been developed to provide the most appropriate ABO component for these patients. For major incompatibility, with recipient anti-A or anti-B directed against donor red cells, it is necessary to use recipient-type RBC units until ABO antibodies are no longer detectable.<sup>10,14,15</sup> For minor incompatibility, recipient-type plasma and platelets are necessary until the recipient's red cells are no longer detectable. Donor-type RBC units can be used immediately after HSCT for minor incompatibility.<sup>10,14,15</sup> For a patient with both major and minor incompatibility ("two-way" incompatibility), group O RBC units and group AB plasma and platelets should be used until offending antibodies and cells are no longer detectable. Tables 31-1 and 31-2 summarize major and minor incompatibility by blood group and provide a transfusion protocol for ABO-mismatched HSCT.<sup>10,14,15</sup> Table 31-2 provides a suitable protocol to follow.

### Alloimmunization to Red Cell Antigens

Despite significant immunosuppression, patients with malignancies may still mount immune responses to "foreign" red cell antigens.<sup>16</sup> This not only complicates transfusion, but

also may increase the risk for hemolysis in patients undergoing allogeneic HSCT.<sup>17</sup> Rates for red cell alloimmunization have been most extensively studied in patients with hematologic malignancies undergoing HSCT and range from 2% to 8% in these populations.<sup>18,19</sup> Other retrospective or prospective studies are necessary to better define the red cell alloimmunization rate for patients with solid tumors and other oncologic problems.

### Alternatives to Allogeneic Red Cell Transfusion

For patients unwilling, or unable, to undergo allogeneic transfusion, several alternatives are available (see Chapter 37). In theory, red cell alternatives should reduce infectious and immunologic complications of allogeneic transfusion while providing similar therapeutic benefit. Several perioperative techniques have been developed to curb allogeneic blood use in the operative setting (see Chapter 38). Acute normovolemic hemodilution (ANH) involves the removal of whole blood immediately before a surgical procedure with crystalloid or colloid volume replacement.<sup>20</sup> If bleeding occurs, the blood is readily available for reinfusion at the bedside. ANH has been shown to reduce the need for allogeneic transfusion in multiple studies.<sup>20</sup> Intraoperative blood recovery—a process whereby shed whole blood is centrifuged, washed of contaminants and debris, and then reinfused to the patient—is also of benefit in reducing allogeneic blood usage.<sup>21</sup> Although some concerns about promotion of tumor cell metastasis with the use of blood recovery and reinfusion have been raised, several trials conducted in the setting of surgery for hepatobiliary and genitourinary cancers have shown no evidence of decreased survival in these patients.<sup>22-24</sup> Furthermore, irradiation of the recovered blood has also been shown to prevent poor outcome in oncology patients undergoing surgery.<sup>25</sup> The use of preoperatively donated autologous whole blood can also be considered, although this is often not feasible for oncology patients who are too anemic and who can require surgery at unpredictable times. Despite the irregular scheduling of oncologic surgery, autologous whole blood donation has been successfully utilized for patients with prostate cancer and

**Table 31-1.** Compatibility by ABO Group in Hematopoietic Stem Cell Transplantation

| Recipient ABO Blood Group | Donor ABO Blood Group |    |    | AB |
|---------------------------|-----------------------|----|----|----|
|                           | O                     | A  | B  |    |
| O                         | C                     | M  | M  | M  |
| A                         | m                     | C  | Mm | M  |
| B                         | m                     | mM | C  | M  |
| AB                        | m                     | m  | m  | C  |

C = compatible; M = major incompatibility; m = minor incompatibility; mM = both major and minor incompatibility ("two-way" incompatibility).

**Table 31-2.** Transfusion Protocol for HSCT Patients

| Recipient | Donor O  |              |                | Donor A  |              |                | Donor B  |              |                | Donor AB |              |                |
|-----------|----------|--------------|----------------|----------|--------------|----------------|----------|--------------|----------------|----------|--------------|----------------|
|           | RBC type | FFP/PLT type | 2nd choice PLT | RBC type | FFP/PLT type | 2nd choice PLT | RBC type | FFP/PLT type | 2nd choice PLT | RBC type | FFP/PLT type | 2nd choice PLT |
| O         | O        | O            | A or           | O        | A            | O              | O        | B            | O              | O        | AB           | A              |
| A         | O        | A            | O              | A        | A            | O              | O        | AB           | A              | A        | AB           | A              |
| B         | O        | B            | O              | O        | AB           | A              | B        | B            | O              | B        | AB           | B              |
| AB        | O        | AB           | A              | A        | AB           | A              | B        | AB           | B              | AB       | AB           | A or B         |

NOTE: Rh positive recipients with Rh negative donors receive Rh negative products. Rh negative recipients with Rh positive donors receive Rh positive products.

gynecologic malignancies.<sup>26,27</sup> It is recommended, however, that the use of preoperative autologous donation be restricted to oncology patients undergoing major surgery, because it is not necessarily a cost-effective therapy for all patients.<sup>28</sup>

The routine administration of erythropoietin-stimulating agents (ESAs) to patients with cancer-related anemia was a strategy developed to reduce allogeneic transfusion within oncology populations.<sup>29</sup> However, the chronic use of ESAs for anemia in patients with cancer, as well as for those with other critical illnesses, has come under scrutiny. Several large trials have demonstrated that ESA administration may lead to decreased patient survival and increased risk for thrombosis.<sup>30-32</sup> It has been postulated that erythropoietin administration accelerates tumor growth, accounting for decreased survival trends.<sup>33</sup> It has also been found that ESAs do not necessarily significantly decrease the need for allogeneic transfusion in critically ill patients.<sup>30-32,34</sup> The results of these trials prompted the Food and Drug Administration (FDA) to issue a “black box warning” for the use of ESAs in the setting of cancer-related anemia.<sup>35</sup> More data and clinical trials will be necessary to ascertain the ultimate safety and efficacy profile for the use of ESAs in oncology patients.

**Component Modification: Irradiation and Leukocyte Reduction**

Because of their immunosuppressed state, many oncology patients are at high risk for transfusion-associated graft-vs-host disease (TA-GVHD), wherein donor lymphocytes generate a profound

immune response against the recipient’s cells.<sup>36</sup> The causes and manifestations of TA-GVHD are discussed below (see Adverse Reactions to Blood Transfusion). Prevention of TA-GVHD can be accomplished with gamma irradiation of cellular blood components.<sup>36</sup> Table 31-3 summarizes disorders for which gamma irradiation is prudent.<sup>37</sup> Some within the field of transfusion medicine have argued for a policy of universal blood component irradiation. The process has few side effects and broad application of irradiation may help to eliminate TA-GVHD in the occasional patient who is at risk for TA-GVHD, but whose high-risk status is not known to the physicians or other caregivers. The practicality and cost-effectiveness of such a policy has not been determined.<sup>38</sup> Irradiation of red cells does have the disadvantage of inducing a red cell membrane potassium leak, which shortens the shelf life of the RBC unit to no more than 28 days from the date of irradiation.

In addition to irradiation, blood collection centers and blood banks have implemented prestorage leukocyte reduction techniques to improve transfusion outcomes. Leukocyte reduction of blood components is particularly beneficial for oncology patients. By decreasing the absolute numbers of white cells in RBC units, alloimmunization to HLA antigens is reduced. Leukocyte reduction has also had an ameliorating effect on immunologic refractoriness to platelet transfusions, a significant problem for chronically transfused oncology patients.<sup>39,40</sup> Unfortunately, prestorage leukocyte reduction has not shown a benefit in the reduction of red cell antigen alloimmunization.<sup>41</sup> Beyond alloimmunization, leukocyte reduction has the potential

**Table 31-3.** Indications for Gamma Irradiation of Cellular Blood Components

|                         |                                          |                                                         |
|-------------------------|------------------------------------------|---------------------------------------------------------|
| Disease State           | Hematologic malignancies                 | Acute myelogenous leukemia                              |
|                         |                                          | Acute lymphocytic leukemia                              |
|                         |                                          | Allogeneic marrow transplantation                       |
|                         |                                          | Autologous marrow transplantation                       |
|                         |                                          | Myelodysplastic syndrome                                |
|                         |                                          | Hodgkin lymphoma                                        |
|                         |                                          | Non-Hodgkin lymphoma                                    |
|                         | Nonhematologic malignancies              | Glioblastoma multiforme                                 |
|                         |                                          | Neuroblastoma                                           |
|                         |                                          | Rhabdomyosarcoma                                        |
| Immunosuppressed states | Congenital immunodeficiency              |                                                         |
|                         | High-dose chemotherapy (eg, fludarabine) |                                                         |
|                         | Solid organ transplantation              |                                                         |
| Blood Components        | Red cells                                | All components for children <4 months                   |
|                         |                                          | All directed donations                                  |
|                         | Platelets                                | All components for children <4 months                   |
|                         |                                          | All directed donations                                  |
|                         |                                          | All apheresis products, including HLA-matched platelets |
|                         | Granulocytes                             | All granulocyte products                                |

to prevent passage of some transfusion transmitted diseases—cytomegalovirus (CMV) and other pathogens residing within white cells are efficiently filtered via leukocyte reduction.<sup>39</sup> Furthermore, studies have shown a reduction in febrile transfusion reactions associated with leukocyte filtration of blood components.<sup>39</sup> According to the 2007 National Blood Collection and Utilization Survey, approximately 72% of all RBC units in the United States in 2006 were leukocyte reduced, most of them before storage.<sup>42</sup>

## Platelet Transfusion

### Indications

Platelet transfusion for oncology patients is intended to stop or prevent bleeding in the setting of thrombocytopenia. The use of platelet transfusion for acute hemorrhage in the patient with low platelet counts, or receiving antiplatelet medication, is a first-line therapy.<sup>43</sup> Much greater controversy exists, however, in the consideration of platelet transfusion for bleeding prophylaxis. Historically, the practice of providing platelet transfusions on a prophylactic basis arose out of studies involving patients with leukemia who were noted to have decreased bleeding episodes following platelet transfusion.<sup>44</sup> Until the early 1990s, routine bleeding prophylaxis included platelet transfusions for oncology patients with platelet counts less than 20,000/ $\mu$ L.<sup>45</sup> However, as clinical experience and evidence mounted, it was found that oncology patients could tolerate much lower platelet counts without development of severe bleeding. A number of clinical trials, beginning in 1991, demonstrated that spontaneous hemorrhage was unlikely even at platelet counts as low as 5000 to 10,000/ $\mu$ L. Bleeding episodes and morbidity in these patients were no worse than for those individuals transfused at higher “trigger” platelet counts.<sup>46–51</sup> These studies also found that stringent transfusion criteria led to a significant reduction in transfused platelet products, thereby reducing the risks associated with chronic transfusion exposure.

The evidence provided by these clinical trials led to the implementation of more conservative platelet transfusion triggers. Most oncology and hematology guidelines now recommend platelet transfusion only in patients with platelet counts less than 10,000/ $\mu$ L in the absence of bleeding.<sup>52,53</sup> It is important to recognize that clinical history, presentation, and comorbid states influence the decision to transfuse platelets. For instance, a patient undergoing a major surgical procedure or one who has a history of platelet dysfunction may require higher baseline platelet counts or more frequent platelet transfusion. For patients undergoing major invasive procedures (eg, central venous catheter placement before HPC collection) goals of platelet counts of 40,000 to 50,000/ $\mu$ L are an appropriate target, while procedures such as marrow aspiration can be safely performed at platelet counts less than 20,000/ $\mu$ L.<sup>54</sup>

Of note, although clinical trials have shown no increased bleeding complications associated with stringent platelet

transfusion criteria, a recent study suggests that persistent and profound thrombocytopenia leads to greater patient mortality. This retrospective analysis of patients following HSCT showed greater risk for mortality for those individuals who received platelets according to stringent (ie, <10,000/ $\mu$ L) criteria.<sup>55</sup> Mortality in these cases, not associated with bleeding episodes, may imply some other protective benefit to increased platelet counts in oncology patients. Prospective studies on long-term patient survival and outcome with conservative approaches to platelet transfusion may yet again change the landscape for the treatment of thrombocytopenic oncology patients.

### Selection of ABO Group and Rh Type for Platelet Transfusion

The ABO antigens are expressed on several platelet proteins and lipids, although the interactions between these antigens and circulating host antibodies do not mediate clinically significant transfusion reactions.<sup>56</sup> Thus, most blood banks will transfuse ABO-mismatched platelets to adults without significant concern for incompatibility. However, for the oncology patient, the use of ABO-mismatched platelet products could result in poor outcome and decreased therapeutic benefit. For instance, a form of platelet refractoriness is mediated by ABO antibodies, with mismatched platelets cleared from circulation minutes to hours after infusion.<sup>56</sup> Furthermore, there is some evidence to suggest that ABO incompatibility can promote the development of HLA alloimmunization in multitransfused patients.<sup>56</sup> Another concern is the possibility for hemolytic transfusion reactions secondary to high-titer or high-avidity ABO antibodies present in the plasma fraction of platelet pools. Although rare, there are several case reports of high-titer ABO antibodies mediating severe hemolysis in patients with hematologic and oncologic diseases receiving apheresis platelet products.<sup>57–60</sup> Children in particular may demonstrate clinically significant hemolysis to ABO-incompatible platelet transfusions given their small blood volume. Therefore, it is advisable that oncology patients with low platelet counts receive ABO-compatible platelet products if at all feasible. For populations undergoing HSCT, other considerations regarding major and minor mismatches are also relevant. Table 31-2 summarizes guidelines for platelet transfusion therapy in the peritransplant period.

Although there are no Rh antigens on platelets, the concern for Rh alloimmunization during platelet transfusion arises secondary to the presence of residual D-antigen-positive red cells in platelet products collected from Rh-positive donors. Even a few milliliters of red cells are sufficient to cause alloimmunization in Rh-negative recipients, because of the immunogenicity of the D-antigen. There is evidence to suggest that, in part due to immunosuppression, both adult and pediatric patients with hematologic malignancies are unlikely to form anti-D responses to Rh-incompatible platelet transfusions.<sup>61,62</sup> Thus, the provision of Rh-incompatible platelet products is likely a safe procedure and unlikely to cause D alloimmunization in oncology populations. Nonetheless, it is still advisable to prevent Rh

alloimmunization in children and females of childbearing potential, because of the consequences of anti-D development in these populations. Alloimmunization can be prevented with a dose of 250 IU (50 µg) of intravenous Rh Immune Globulin provided within 72 hours of D antigen exposure.<sup>63</sup>

Platelet Refractoriness and Alloimmunization

In the chronically transfused oncology patient, increases in platelet count may be lower than expected following transfusion. This phenomenon, known as platelet refractoriness, can be attributed to both immune and nonimmune causes. In oncology patients, approximately 70% to 80% of cases of platelet refractoriness are caused by nonimmunologic factors.<sup>43</sup> Table 31-4 provides a list of the most common variables associated with nonimmune platelet refractoriness. For the patient with non-immunologic platelet refractoriness, few clinical management options exist. Addressing the underlying cause (eg, splenectomy for hypersplenism, removal of an offending medication) can often alleviate refractoriness. For the acutely bleeding patient or for hemorrhage prophylaxis, strategies of continuous platelet infusion (“platelet drip”) have been attempted with moderate success.<sup>54,64</sup> The “platelet drip,” wherein a dose of platelet concentrate is infused slowly over a 4-hour period, is intended to provide an ongoing source of platelets to maintain vascular integrity, while addressing the practical concern of blood bank platelet inventory conservation.

An underlying antibody to platelet antigens is most often responsible for the remaining 20% to 30% of platelet refractoriness in oncology patients. Platelets express Class I HLA and numerous other platelet-specific antigens. Most commonly, HLA antigens mediate immunologic platelet refractoriness, although the chronically transfused patient may develop alloantibodies to any platelet antigen.<sup>43,65</sup> Treatment strategies for immune-mediated platelet refractoriness center on providing antigen-negative components. The first and simplest strategy for managing immune platelet refractoriness is to provide ABO-compatible units. The next option would be

to obtain crossmatch-compatible platelets from the hospital blood bank or donor center. This process involves crossmatching of patient serum against a variety of donor platelets and selection of those products for which no agglutination reaction is observed.<sup>66</sup> Platelet count responses to crossmatch-compatible platelets may be sustainable for 2 to 3 days, but this is also dependent on the age of the unit and other underlying clinical factors.

If the majority of tested donors are incompatible with recipient serum, HLA typing of the recipient and provision of HLA antigen-matched platelet products is likely necessary.<sup>43,65</sup> Polymorphisms of HLA Class I antigens can complicate compatibility testing and make the process of finding fully matched donors very difficult. The Duquesnoy grading system was developed to allow for the most advantageous use of partially mismatched donations.<sup>67</sup> Newly developed computerized algorithms, based on characterization of HLA polymorphisms, are paving the way for broader matches that may help reduce the need to provide partially incompatible platelet products.<sup>68</sup>

Crossmatch-compatible platelets have been proven to be as efficacious as HLA-matched platelets in raising platelet counts in alloimmunized patients.<sup>43,65,66</sup> Thus, the decision to provide HLA-matched platelets should depend upon factors such as quality of the HLA match, severity of alloimmunization, and availability of crossmatched platelet products. In many cases, crossmatch-compatible platelets can be more quickly and easily obtained and are available at lower cost than HLA-matched products.

The best strategy to reduce immune-mediated platelet refractoriness is prevention of HLA and platelet antigen exposures. The trend toward universal leukocyte reduction has helped reduce the incidence of alloimmune platelet refractoriness by limiting exposure to HLA antigens.<sup>40</sup> Conservative transfusion strategies also help to prevent exposure to various HLA and platelet-specific antigens and may play a role in reducing the frequency of alloimmunization. Unfortunately, once alloimmunization has occurred, immune-modulation with corticosteroids, plasmapheresis, and intravenous immune globulin have been of little benefit.<sup>69</sup>

Table 31-4. Most Common Causes of Refractoriness to Platelet Transfusion

| Category                      | Cause                                                             |
|-------------------------------|-------------------------------------------------------------------|
| Nonimmunologic refractoriness | Acute bleeding/consumption                                        |
|                               | Fever (any cause)                                                 |
|                               | Infection                                                         |
|                               | Medications (eg, amphotericin B)                                  |
|                               | Microangiopathy or disseminated intravascular coagulation         |
|                               | Splenomegaly                                                      |
| Immunologic refractoriness    | ABO-mismatched transfusion                                        |
|                               | HLA and/or platelet-specific alloantibodies                       |
|                               | Autoimmune thrombocytopenia (eg, immune thrombocytopenic purpura) |

Alternatives to Platelet Transfusion

For patients unwilling to undergo platelet transfusion, or for severe platelet refractoriness, several medical therapies can aid in bleeding episodes. A commonly prescribed drug, 1-deamino-8-D-arginine vasopressin (DDAVP), acts to stimulate the release of von Willebrand factor from endothelial cells, which can enhance platelet activity even at very low platelet counts.<sup>70</sup> DDAVP is also of proven benefit for platelet dysfunction and bleeding associated with uremia.<sup>71</sup> Antifibrinolytic therapies have also been employed as an adjunct to platelet transfusions for the bleeding patient. Medications such as aminocaproic acid, tranexamic acid, and aprotinin have all been successfully used to reduce hemorrhage and allogeneic transfusion requirements in the bleeding patient.<sup>72,73</sup>

Chemical and cytokine-based stimulation of the marrow to endogenously increase platelet production has also been attempted. Agents such as thrombopoietin (TPO) and megakaryocyte growth factors have been synthesized, but clinical trials with some of these drugs led to the development of neutralizing antibodies and thrombocytopenia in recipients.<sup>74,75</sup> TPO and TPO-like growth factors are finishing clinical trials and may be considered for FDA licensure in the near future. One such agent, AMG531 (Nplate, Amgen, Thousand Oaks, CA), has been licensed by the FDA. Interleukin (IL)-11, a cytokine that drives megakaryocyte production and division, also has been approved for use in thrombocytopenia.<sup>74,75</sup> The development of other similar cytokine agents is currently under way.<sup>74,75</sup>

### Selection of Platelet Products

Platelet products in the United States, Canada, and Europe can be prepared in several ways. In the past, the majority of platelet units available for transfusion were derived, via centrifugation, from whole blood.<sup>43</sup> These individual units are pooled in groups of four to five to yield a product intended to raise platelet counts by approximately 50,000/ $\mu$ L. Thus, platelet pools consist of individual units from multiple donors. Since the 1970s, apheresis techniques have been developed to allow for the collection of full doses of platelet products from one donor.<sup>76</sup> Apheresis platelets have grown in popularity and usage, and provide a platelet dose virtually equivalent to pooled platelets.<sup>43</sup> Thus, the decision to use platelet pools or platelets from one donor relates more to concerns about exposures to multiple donors, risks for alloimmunization, and platelet quality rather than increases in baseline platelet count.

The primary consequence of transfusions obtained from multiple donors is an increased risk for transfusion-transmitted disease.<sup>77</sup> For the heavily immunosuppressed oncology patient, this fact alone would seemingly favor the use of apheresis platelets. However, with more rigorous donor screening and sensitive nucleic acid testing, the risk of transfusion-transmitted disease is virtually the same between platelet product options. In addition, even with the use of apheresis products, chronically transfused oncology patients are likely to be exposed to a multitude of donors over the course of their disease. Thus, the use of one apheresis platelet unit in an overall transfusion strategy has minimal benefit in reducing infectious disease risks. Thus, from an infectious disease standpoint, apheresis platelets are not necessarily preferred.

Another consideration in exposure to multiple donors is increased risk for platelet alloimmunization. In the era before leukocyte reduction, this was a significant concern for pooled products. However, with the advent of leukocyte reduction, a patient is as likely to become alloimmunized to a platelet pool as to a platelet product from an individual donor.<sup>40</sup> Thus, there is minimal advantage in using apheresis platelets to prevent alloimmunization. Once a patient has become alloimmunized, the use of platelet pools may be advantageous until crossmatch-compatible or HLA-matched units are available. In this scenario, an

alloimmunized thrombocytopenic patient may be more likely to respond to one of the four or five donors whose units constitute a platelet pool. For the alloimmunized patient, apheresis platelets should be employed only if the unit is crossmatch-compatible or HLA-matched.<sup>43</sup>

The quality of platelet products does differ somewhat between pooled and apheresis units. Markers of platelet activation, such as P-selectin, are increased in whole-blood-derived platelet concentrates as compared to apheresis platelets.<sup>78</sup> These changes, however, have not correlated with poor in-vivo platelet recovery. Thus, current evidence does not suggest that apheresis platelets are a more viable platelet option. From the standpoint of adverse reactions to platelet transfusion, large trials have revealed no difference in adverse events between apheresis and whole-blood-derived products.<sup>79</sup> Bacterial contamination does remain an outstanding concern for pooled platelets given the number of donors and various manipulations of the unit before issuance of the final product. Methods to address the contamination of pooled products, such as prestorage pooling with sampling for bacterial cultures, may help to identify contaminated units.<sup>80</sup> By comparison, apheresis products do offer a lower risk for bacterial contamination, but the risk for contamination has not been completely eliminated.<sup>81,82</sup> Indeed, 7-day apheresis platelets have been reported to be associated with greater bacterial contamination than 5-day apheresis platelets. Thus, from the standpoint of platelet quality, apheresis platelets seem to offer little advantage when compared to platelet concentrate pools.

### Platelet Product Modification: Irradiation, Leukocyte Reduction, and Volume Reduction

As is the case with RBC units, platelets can be modified to maximize safety for oncology patients. Despite leukocyte reduction, platelet units contain small amounts of white cells. Thus, patients at risk for TA-GVHD should receive irradiated platelets.<sup>36</sup> Table 31-3 summarizes those malignant conditions for which gamma irradiation is appropriate. As mentioned previously, leukocyte reduction of platelet products helps to reduce alloimmunization and refractoriness.<sup>40</sup> According to the 2007 National Blood Collection and Utilization Survey, approximately 40% of all whole-blood-derived platelet units were leukocyte reduced, and essentially all apheresis platelets were leukocyte reduced.<sup>42</sup> The movement toward universal leukocyte reduction in the United States should help ease the burden of alloimmunization in coming years.

Volume reduction of platelet products via centrifugation and resuspension in saline should be considered for oncology patients who have experienced severe allergic and/or anaphylactic reactions to platelet products.<sup>83</sup> Volume reduction is also efficient at removing the plasma fraction of platelet products to reduce the risk of hemolysis associated with ABO-incompatible transfusion.<sup>83</sup> Volume reduction and saline resuspension should be considered for ABO-mismatched platelet transfusions in infants and children with small blood volumes.

## Plasma and Cryoprecipitated AHF Transfusion

### Indications, Selection of ABO Group, and Product Modification

Plasma transfusions should be used to stop or prevent bleeding in patients with coagulopathy caused by vitamin K antagonists, factor consumption, or liver disease.<sup>84,85</sup> Cryoprecipitated AHF can be used to treat bleeding associated with uremia or Factor XIII deficiency associated with severe GVHD.<sup>71,86</sup> Plasma should not be used as the sole fluid for volume replacement because of limited supplies and the risks associated with blood transfusion. For the majority of oncology patients, no specific additional consideration is given to selection of ABO group for plasma or cryoprecipitate so long as the unit is ABO compatible with the recipient. Donor and recipient Rh types are not a consideration for infusion of plasma or cryoprecipitate. As mentioned previously, for patients who have undergone allogeneic HSCT, the choice of ABO group can be complex and should be based upon consideration of major and minor mismatches. Table 31-2 summarizes guidelines for plasma transfusion therapy in the peri-transplant period; guidelines for cryoprecipitated AHF are identical to those for plasma. Plasma and cryoprecipitated AHF are maintained at temperatures below  $-20^{\circ}\text{C}$ ; because they are acellular, there is no need for gamma irradiation.

### Alternatives to Plasma Transfusion

For some oncology patients, plasma transfusion may be contraindicated, may be ineffective, or (in the setting of acute hemorrhage) may not provide rapid enough reversal of coagulopathic states. For these conditions, there are several alternatives to plasma products, mostly consisting of recombinant coagulation factors. Among the most commonly used agents for acute moderate to severe bleeding is recombinant activated Factor VII, a potent activator of the coagulation cascade.<sup>87</sup> Successful use of Factor VIIa has been reported to help control massive bleeding in a variety of oncology patients.<sup>88-91</sup> There is also a role for use of Factor VIIa as a bypass agent for those oncology patients who acquire inhibitors to circulating coagulation factors, mainly Factor VIII.<sup>92</sup> Factor VIIa failures in bleeding oncology patients have also been reported; a patient with refractory gastrointestinal bleeding associated with GVHD following HSCT showed no improvement of hemorrhage following Factor VIIa administration.<sup>93</sup>

The instances of Factor VIIa usage cited above highlight the difficulty of recommending broad application of this therapy to the bleeding oncology patient. The most significant problem with making an evidence-based recommendation is that the vast majority of uses of Factor VIIa in the literature are in the form of case reports. Few adequate clinical trials have been conducted to determine the safety and efficacy of this drug in oncology populations. Furthermore, appropriate dosing regimens are imprecise and mostly based on data gathered in trials performed in other critically ill patient populations. Factor VIIa also carries a number of risks, chief of which is the possibility for development

of severe or even fatal thromboembolism.<sup>94</sup> Thus, Factor VIIa may be considered as an alternative to plasma infusion, but should be used only after other interventions have failed.

### Intravenous Immune Globulin

Intravenous immune globulin (IVIG) has become a very useful product in the management of oncology patients.<sup>95</sup> Consisting of human gamma globulins, IVIG has been used to provide passive immunity in highly immunosuppressed individuals. IVIG has also been successfully used as immunomodulatory therapy for patients with conditions such as immune thrombocytopenic purpura (ITP).<sup>96,97</sup> For recipients of HPC products, IVIG has been associated with improved immune defense against pathogens such as CMV and has helped to decrease the complications of acute GVHD.<sup>98,99</sup> The benefit from use of IVIG for chronic GVHD and infection prophylaxis in marrow transplantation is not clear and warrants further study.<sup>99</sup>

IVIG infusion is usually tolerated without significant adverse events in oncology patients. The common side effects of administration include myalgias, headache, fever, and fatigue.<sup>100</sup> A rare but serious complication of IVIG administration is acute renal failure seen with particular formulations using sucrose as a globulin stabilizer.<sup>100</sup> Thus, oncology patients with chronic renal insufficiency should be closely monitored during IVIG administration. Current IVIG products have not transmitted hepatitis B, hepatitis C, or human immunodeficiency virus; there are rare case reports of transmission of viruses such as parvovirus B19, a serious pathogen for oncology patients.<sup>101</sup> Plasma is now screened for this virus before being pooled for fractionation.

## Granulocyte Transfusion

### Indications

Granulocyte transfusion is a therapeutic option used almost exclusively in oncology patients. Classically, granulocyte infusions have been used to treat severe, antibiotic-refractory bacterial or fungal infections in patients with an absolute neutrophil count of less than  $500/\mu\text{L}$ . Only patients with a reasonable chance at sustainable marrow recovery after resolution of the underlying infection are candidates for granulocyte products.<sup>102</sup> Granulocyte infusion may be useful for patients who have received ablative chemotherapy regimens and who are not able to mount an adequate cellular response to infection. In general, to overcome a significant bacterial or fungal infection, humans should produce  $2$  to  $3 \times 10^{11}$  polymorphonuclear cells per day, numbers that often cannot be achieved for the immunosuppressed oncology patient.<sup>103</sup> The transfusion of exogenous granulocytes can provide additional innate immunity. Doses of  $1 \times 10^{11}$  granulocytes per square meter of body surface area have been reported to increase neutrophil counts by 1000 to 2000/ $\mu\text{L}$  are higher.<sup>103</sup>

In the past, the most significant barrier to the use of granulocytes was obtaining an adequate dose from a healthy donor. Trials performed in the 1970s and 1980s, using whole blood filtration

and centrifugation techniques, showed little clinical benefit likely secondary to suboptimal granulocyte doses.<sup>102</sup> By the mid-1980s granulocyte infusion therapy was largely abandoned. The technique gained renewed interest in the early 1990s primarily because of the development of granulocyte colony-stimulating factor (G-CSF).<sup>104</sup> The administration of G-CSF to healthy donors allowed for nearly 10-fold increases in granulocyte collection yield. G-CSF has led to higher granulocyte doses and has the added benefit of promoting persistent elevation in recipient white count after infusion, attributable to mobilization of both mature granulocytes and earlier myeloid precursors in donor products.<sup>102</sup> Improvements in apheresis technology have also helped to increase white cell collection efficiency.

Over the last 10 to 15 years, a number of trials have been performed to evaluate the safety and efficacy of granulocyte transfusion using G-CSF stimulation in healthy donors. Studies performed in both adult and pediatric populations have found that granulocyte transfusion leads to significant improvement in patients with fungal infections refractory to antimicrobial drugs.<sup>105–110</sup> Granulocyte doses in these studies were routinely 5 to  $10 \times 10^{10}$  with resultant increases in peripheral white count of 2000 to 5000/ $\mu\text{L}$ . There is also a growing body of literature on the prophylactic use of granulocyte transfusion to prevent onset or recurrence of severe infection.<sup>106,111–113</sup> While this therapy may have some efficacy in prevention of fungal infection recurrence, the role of granulocytes as an agent for primary infection prophylaxis remains unclear. To determine the true efficacy of granulocyte transfusion in these settings, future randomized, controlled trials will be necessary.<sup>102</sup>

### Selection of ABO Group and Donor Preparation

Healthy donors who qualify for granulocyte transfusion should be ABO-compatible with the intended recipient; products must be crossmatched before infusion because of high red cell content. A popular granulocyte mobilization regimen used in donors consists of the administration of 5 to 10  $\mu\text{g/kg}$  G-CSF subcutaneously, in combination with 8 mg oral dexamethasone, both administered approximately 12 hours before leukapheresis.<sup>114</sup> Granulocyte collections can be performed serially over 4 to 5 days to yield multiple doses for neutropenic patients. For granulocyte donors, side effects are usually mild and include bone pain, headache, fatigue, and fluid retention caused by G-CSF administration and apheresis.<sup>102,110,114</sup>

### Alloimmunization

The large number of white cells, red cells, and platelets contained within a granulocyte product increases the potential for red cell, HLA, and platelet antigen alloimmunization in oncology patients.<sup>115</sup> Unfortunately, most donor centers do not have the time to match donors and recipients according to HLA antigen expression given the urgent need to supply the granulocyte product. There is evidence to show that those patients previously alloimmunized to HLA antigens via chronic transfusion demonstrate reduced response to granulocyte infusion.<sup>116</sup> Thus, the

expectation for successful response to granulocyte therapy should be tempered in an individual who has previously demonstrated HLA alloimmunization. For these patients, HLA-antigen matching may be necessary to improve outcome of granulocyte transfusion.

### Alternatives to Granulocyte Transfusion

The decision to initiate granulocyte transfusion usually represents failure of other forms of therapy. In these cases, few other treatment options exist. The administration of G-CSF to the neutropenic patient may aid in the production of endogenous granulocytes and is a potential treatment strategy for infection prophylaxis.<sup>117</sup> For a reduction in fungal infection recurrence, at least one study has shown that the antifungal agent voriconazole is successful in preventing microorganism reactivation.<sup>118</sup>

### Granulocyte Product Storage, Modification, and Infusion

Granulocytes must be stored at 20 to 24°C without agitation.<sup>119</sup> Granulocytes must be infused within 24 hours of collection because of the limited life span of neutrophils and preferably should be given within 6 to 8 hours of collection. Before issuance, the granulocyte product should undergo gamma irradiation to prevent recipient TA-GVHD.<sup>120</sup> It is important to remind clinical staff that granulocytes should be infused through a 170- to 260-micron filter, but that leukocyte reduction filters should *never* be used.<sup>121</sup>

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## Adverse Reactions to Blood Transfusion

Oncology patients undergoing transfusion therapy are subject to the same set of adverse reactions as any patient receiving blood components. However, in the oncology patient, discerning between an adverse transfusion event and worsening of an underlying condition can be difficult. Furthermore, the immunosuppressed oncology patient may be at greater risk for transfusion-transmitted infections and TA-GVHD when compared with immune-competent hosts. This section briefly explores those adverse reactions to transfusion that are most relevant to the oncology patient.

### Transfusion-Associated Graft-vs-Host Disease

The treatment of many malignant diseases relies heavily upon immunosuppressive drugs (eg, purine analogs such as fludarabine) and high-dose ablative chemotherapy. These therapies place oncology patients at particularly high risk for development of TA-GVHD.<sup>122,123</sup> In addition to immune suppression, transfusion of blood from HLA homologous donors places the recipient at increased risk for TA-GVHD.<sup>124</sup> This can be particularly problematic for HLA antigen-matched blood or directed donations from family members. TA-GVHD is common, with estimates that up to 1% of patients with hematologic malignancies can develop this condition at some point during their care.<sup>122</sup> In all cases of TA-GVHD, depletion of T-cell immunity allows donor lymphocytes to generate immune responses



against host tissue. TA-GVHD usually manifests within 2 weeks of transfusion as acutaneous rash with fever, diarrhea, liver function abnormalities, nausea, and vomiting.<sup>125</sup> Unfortunately, after the onset of TA-GVHD, there are few therapeutic options. Administration of corticosteroids, antithymocyte globulin, and cyclosporine have been attempted but to date have shown little efficacy. A diagnosis of TA-GVHD carries mortality approaching 100%.<sup>125</sup>

As mentioned previously, the best strategy to eliminate TA-GVHD is to prevent its occurrence. The most successful and proven mode of prevention is gamma irradiation of blood components. The AABB specifies a radiation dose of 2500 cGy directed at the center of the blood component with a minimum dosage of 1500 cGy achieved in all portions of the unit.<sup>126</sup> Only cellular components (ie, RBCs, platelets, and granulocytes) require irradiation. Leukocyte reduction is thought to contribute to prevention of GVHD.<sup>122</sup> However, leukocyte reduction must not be relied on as the sole modality for GVHD prophylaxis because the residual white counts at which GVHD is prevented are unknown.<sup>122</sup> If such cellular components are given to susceptible patients, the component(s) must be irradiated.

### Transfusion-Transmitted Diseases

Cytomegalovirus (CMV) is among the most problematic of the transfusion-transmitted agents for the oncology patient. It is a highly prevalent virus, with data indicating that 40% to 50% of healthy adults carry CMV antibodies.<sup>127</sup> Within blood donor populations, CMV has been detected in mononuclear cells of both seropositive *and* seronegative individuals, making the identification of true CMV-negative donors difficult.<sup>128</sup> For the immune-competent host, CMV can usually be controlled through various immune mechanisms and is rarely clinically significant. For the oncology patient, however, CMV infection can have serious consequences including pneumonia, gastrointestinal inflammation/infection, and delayed engraftment of myeloid cell lines for recipients of HSCT products. For the oncology patient who has never been exposed to CMV or for the severely immunosuppressed individual, there are several options for blood transfusion. CMV-seronegative blood components can be obtained from blood donor centers, although ABO-compatible components may be hard to find as the majority of urban adults are already CMV positive. Additionally, as mentioned above, even CMV-seronegative donors may harbor residual virus within their white cells. Leukocyte reduction of blood components is thus an excellent alternative to reduce the risk of CMV transmission. Studies have shown that leukocyte removal is equally efficient as CMV-seronegative blood components for prevention of viral transmission.<sup>129,130</sup> Despite these data, several other studies suggest that CMV virus transmission can occur even with leukocyte reduction efforts.<sup>131,132</sup> Currently, there is general consensus that in the absence of blood components that are definitively seronegative, leukocyte reduction is an acceptable alternative for reducing transmission of CMV to oncology patients.

Parvovirus B19, a nonenveloped, single-stranded DNA virus, infects erythroid precursors, leading to suppression of red cell production.<sup>133</sup> For immunosuppressed patients, parvovirus infection can result in severe erythroid hyperplasia and the development of red cell aplasia.<sup>133</sup> Fortunately, active parvovirus is not found in many healthy blood donors. Two recent European studies showed that less than 1% of blood donors actively carry parvovirus in peripheral blood as detected by polymerase chain reaction (PCR) methods.<sup>134-136</sup> There have been only occasional case reports documenting transmission of parvovirus B19 to hematology and oncology patients. In these cases, patients develop characteristic red cell aplasia and often remain chronically transfusion dependent.<sup>137-139</sup> The rarity of reports of transmission of this virus may indicate low infectivity via transfusion, or may represent lack of disease recognition on the part of clinicians and transfusion service personnel. A large retrospective study showed that the actual risk of parvovirus transmission to oncology patients is quite low. In this study, 1% of all blood components from a hospital blood bank tested positive for parvovirus DNA by PCR. Of all virus-contaminated units, 14 were received by patients with underlying hematologic malignancies and none developed signs, symptoms, or laboratory findings consistent with parvovirus infection.<sup>140</sup> Because of the low rates of transmission, the cost efficacy and clinical utility of screening all blood donors for this organism has yet to be determined.<sup>134,136</sup> In order to be most prudent in the application of parvovirus testing, screening of units intended for high-risk patients has been suggested as a strategy for prevention of disease transmission.<sup>141</sup>

West Nile virus (WNV) is a mosquito-borne pathogen that emerged in 1999 as a significant cause of morbidity and mortality in the United States. The presentation of WNV can be highly variable; patients can be completely asymptomatic or experience a spectrum of symptoms ranging from mild fever and fatigue to severe encephalitis.<sup>142</sup> In addition to mosquito-spread illness, transmission of WNV via blood transfusion has been repeatedly documented.<sup>143</sup> Although any transfusion recipient is susceptible to WNV infection, there is some evidence to suggest that immunosuppressed individuals, such as those undergoing chemotherapy and transplantation, are at higher risk for developing some of the more severe neurologic complications of WNV infection.<sup>144</sup> United States and Canadian blood centers have implemented routine nucleic acid testing of blood donors using advanced algorithms to match the seasonal nature of virus infectivity.<sup>145</sup> Current screening techniques should help to limit the exposure of oncology patients to WNV.

*Trypanosoma cruzi*, transmitted via the bite of the reduviid bug, is the blood-borne parasite responsible for Chagas' disease. After initial exposure of the host to the parasite, Chagas' disease manifests as a transient acute illness.<sup>146</sup> Over a number of years, the trypanosome mediates ongoing destruction of multiple organ systems, causing chronic cardiac, gastrointestinal, and neurologic disease resistant to treatment with current antimicrobial drugs.<sup>146</sup> Chagas' disease is endemic in Latin America, but thou-

sands of individuals in North America also carry the disease.<sup>146</sup> Transmission of *T. cruzi* via transfusion has been reported in several patients in the United States and Canada; most reported cases have occurred in severely immunocompromised oncology patients.<sup>147–150</sup> The seeming exclusivity of transmission to oncology patients is thought to be caused by underlying host immunosuppression.<sup>147</sup> As a result of the growing risk for trypanosome transmission in North America, US and Canadian blood centers have undertaken efforts to broaden laboratory and medical history screening measures to better assess donor risk factors for Chagas' disease.<sup>151,152</sup> As with other pathogens, increased vigilance should help to reduce the devastating effects of trypanosome transmission in oncology patients.

In addition to thorough donor history screening and a battery of serologic and nucleic acid tests, there have been several advancements in the field of pathogen inactivation that could be helpful to oncology patients undergoing transfusion.<sup>153</sup> For instance, there is growing interest in photochemical treatment of blood components involving the addition of the chemical amotosalen with subsequent long-wave ultraviolet irradiation.<sup>154,155</sup> This technique—which causes disruption of pathogen DNA, preventing microorganism activation and proliferation—has shown great efficacy in reducing the infectivity of a number of pathogens harmful to oncology patients including CMV, parvovirus B19, and *T. cruzi*.<sup>154,156,157</sup> If clinical trials and safety data continue to look promising, pathogen inactivation should likely further improve transfusion safety for the oncology patient. As of this writing, amotosalen-treated blood components are not FDA-licensed in the United States.

### Transfusion-Related Acute Lung Injury

Transfusion-related acute lung injury (TRALI) represents the development of noncardiogenic pulmonary edema in patients undergoing transfusion therapy. TRALI has been theorized to be caused by antibodies that activate neutrophils passing through pulmonary vasculature.<sup>158</sup> These antibodies are believed to be directed against HLA antigens and are more frequently found in donor blood than in recipient plasma.<sup>158</sup> Oncology patients, per se, are not at increased risk for TRALI as compared to other patient populations. However, particular blood components used in oncology populations, such as granulocytes, can be associated with increased risks for TRALI. Pulmonary problems associated with granulocyte infusion can be particularly severe for patients who previously have been alloimmunized to HLA antigens. There is also some evidence to suggest that concurrent administration of granulocytes and the antifungal agent amphotericin B can lead to higher rates of pulmonary complications.<sup>159</sup> Thus, it is recommended that granulocyte infusion and amphotericin B therapy be spaced out over time to avoid pulmonary decompensation.

### Acute Hemolytic Transfusion Reactions

Acute intravascular red cell hemolysis wherein red cells are destroyed can be a catastrophic complication of blood transfusion, leading to renal failure, shock, hypotension, and death. As

with TRALI, oncology patients are not necessarily at increased risk for hemolysis because of their underlying disease state. Nonetheless, a series of case reports has documented severe hemolysis occurring in oncology patients receiving ABO-incompatible apheresis platelet products with high-titer ABO agglutinins.<sup>57–60</sup> Thus, it is recommended that oncology patients, particularly children, receive ABO-compatible platelet pools whenever possible. If no other options exist and ABO-incompatible platelets must be provided, volume reduction with removal of incompatible plasma can greatly reduce the risk of hemolysis.<sup>83</sup>

### Febrile Nonhemolytic Transfusion Reactions

Most of the blood components provided to oncology patients can cause rigors, chills, and transient elevation in body temperature. Unfortunately, it is often difficult to distinguish between the signs and symptoms of a febrile nonhemolytic transfusion reaction (FNHTR) and new-onset fever caused by infection in an immunosuppressed patient. Some of the clinical characteristics of FNHTRs may be helpful in distinguishing between these entities. FNHTRs involve an increase in temperature of at least 1°C (1.8°F) during or within 2 hours of the completion of a transfusion.<sup>160</sup> Fevers that begin more than 2 hours after the completion of transfusion are most likely due to another cause. Symptoms of FNHTRs typically appear during the latter half of a transfusion as causative cytokines or agents accumulate in circulation.<sup>160</sup> Despite clues on presentation, FNHTR ultimately remains a diagnosis of exclusion. For the oncology patient who experiences fever during transfusion, a full evaluation by transfusion service personnel and the clinical team is necessary to rule out other causes for temperature elevation.

Another strategy to deal with FNHTRs in oncology populations is to prevent their occurrence. In patients who have had repeated febrile reactions to blood transfusion, an antipyretic agent (eg, acetaminophen) given 30 minutes before transfusion can decrease adverse febrile events.<sup>160</sup> Aspirin or aspirin-containing products should not be given to thrombocytopenic patients. Aspirin acetylates the platelet enzyme cyclooxygenase and this induces a thrombocytopeny, preventing the platelet release reaction. Prestorage leukocyte reduction of blood components has also been shown to reduce the incidence of FNHTRs.<sup>161</sup>

### Transfusion-Associated Immunomodulation

The administration of allogeneic blood components can have consequences for immune function in all patient populations; most notable among these are apparent decreases in cellular immunity. These deleterious immune effects have been reported in several non-oncologic populations wherein transfusion is noted to suppress the immune system.<sup>162,163</sup> The immunomodulatory effects of allogeneic transfusion for oncology patients are slowly being unraveled. Retrospective studies indicate that allogeneic transfusion in cancer patients could lead to immunosuppression, promoting solid tumor recurrence

and increased susceptibility to primary and reactivated viral infection.<sup>164-166</sup> Conversely, pediatric literature suggests that allogeneic transfusion has little negative effect on outcome for children with acute lymphoblastic leukemia; other groups of pediatric oncology patients have not been extensively studied.<sup>167</sup>

Several animal studies have recapitulated the tumor recurrence effects of allogeneic transfusion seen in adults.<sup>168-172</sup> These studies, and others performed *in vitro*, have identified several candidates that may be responsible for the immunosuppressive effects including ubiquitin, fas and fas-ligand, soluble HLA peptides, and other mediators derived from the mononuclear elements of blood.<sup>163,171,173</sup> Particular focus has been placed on the relationship between leukocyte reduction of blood components and their immunomodulatory effects. Prestorage leukocyte reduction appears to reduce the immunosuppressive effects of transfusion, perhaps by prophylactic removal of soluble immune mediators secreted by white cells that would otherwise accrue during storage.<sup>169</sup> Future models and randomized, controlled trials may help clinicians better classify this immunologic phenomenon and address its potential causes.

### Adverse Effects of Hematopoietic Progenitor Cell Infusion

The majority of allogeneic and autologous HPCs are cryopreserved using solutions containing 10% dimethylsulfoxide (DMSO).<sup>174</sup> DMSO is a unique chemical modifier that allows for the controlled freezing and thawing of mononuclear cells without development of membrane lysis.<sup>174</sup> Traditionally, the adverse events associated with HPC infusion have been associated with the DMSO content of the progenitor product. DMSO is a toxic substance and has been linked to fever, nausea, vomiting, and chills during or immediately after HPC infusion; patients receiving fresh, noncryopreserved cells generally experience these symptoms to a much less degree.<sup>175</sup> DMSO has also been linked to pulmonary and cardiovascular problems during HPC infusion including dyspnea, hypotension, bradycardia, and arrhythmia.<sup>174,175</sup> The number of HPC units being infused and the size of the individual receiving the infusion are believed to enhance DMSO toxicity. Small children and those patients receiving multiple HPC units are more susceptible to DMSO-related problems.<sup>174</sup>

As clinical experience and evidence have been compiled, researchers have begun to evaluate other components of the HPC product as they related to infusion toxicity. Several recent studies have correlated adverse events to the numbers of granulocytes present in HPC products.<sup>176,177</sup> These studies found that even after DMSO depletion in the cellular product, 50% to 60% of patients undergoing HPC infusion experienced an adverse event such as fever, rigors, or dyspnea. It is thus possible that the dyspnea associated with HPC infusion is not entirely caused by DMSO, but rather is mediated by granulocytes through TRALI-like mechanisms. These studies argue that a reduction in granulocyte content of the final HPC product leads to better tolerance of infusion. The challenge would be to remove the unwanted granulocytes while leaving behind the lifesaving CD34+ cells.

In addition to the adverse events associated with DMSO and granulocyte content, patients undergoing HPC infusion often experience symptoms similar to those seen after traditional blood component transfusion. TRALI and anaphylactic reactions during autologous and allogeneic HPC infusions have been reported.<sup>178,179</sup> These reactions are rare, but must be considered part of the differential diagnosis for a patient presenting with shortness of breath or cardiovascular complications following HPC infusion.

Bacterial contamination of HPC products, occurring during collection or processing phases, is also of concern.<sup>180</sup> HPCs are routinely cultured and, if bacterially contaminated, are provided to patients in conjunction with prophylactic broad-spectrum antibiotics before infusion. A recent study showed that of 13 patients who received bacterially contaminated HPCs, only one developed evidence of bacteremia.<sup>180</sup> Thus, with appropriate vigilance and antibiotic therapy, bacterial contamination of an HPC product need not be an absolute barrier to infusion.

Many efforts are under way to reduce the toxicity and side effects that are associated with HPC infusion. The largest target has been DMSO; many studies have shown that reducing the content of this additive from 10% to 5% of the final product volume does not negatively affect HPC viability during storage, thawing, or reinfusion.<sup>174</sup> There are also devices undergoing evaluation that can be used to “wash” HPC products, effectively reducing DMSO content before infusion.<sup>181</sup> With regard to DMSO-related symptoms, premedication with antipyretic and antihistamine as medications 30 minutes before infusion may help alleviate unpleasant side effects such as fever and rigors and chills. Adequate field sterilization practices during stem collection and the processing of HPC products in sterile areas with air-controlled fume hoods can also help to reduce risks of bacterial contamination.<sup>182</sup>

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## Summary

Transfusion therapy plays a major role in the care of patients with hematologic or oncologic disorders; however, blood transfusion carries risks for these patients that are generally of less concern for other general hospital populations. For oncology patients, RBC transfusions are given for increased oxygen-carrying capacity, platelets for the cessation or prevention of bleeding due to thrombocytopenia, plasma for coagulopathies, and granulocytes for pervasive bacterial and fungal infections. All of these components can create special problems for oncology patients including TA-GVHD, transfusion-transmitted diseases, alloimmunization to blood cell antigens, pulmonary decompensation, and immunomodulation. Patients undergoing HPC transplantation are a unique group and present complex concerns related to transfusion, including major and minor ABO incompatibility and chimeric blood cells. Transfusion for patients undergoing treatment with cellular therapies requires careful blood component selection. The process of HPC infusion itself carries many risks including DMSO toxicity and hemolytic reactions. In all areas of transfusion therapy, new advances such as pathogen inactivation and synthetic alternatives

to whole blood should help to increase the safety and tolerance of blood component infusion within oncology populations.

## Acknowledgment

The authors acknowledge the outstanding work of the late Margot S. Kruskall, MD, whose original text for the 3rd edition of *Rossi's Principles of Transfusion Medicine* formed the template for this chapter in the 4th edition.

## Disclaimer

C. Tormey has disclosed no conflicts of interest. E. Snyder has disclosed financial relationships with Fenwal, CaridianBCT, Terumo, Pall Corporation.

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Hematopoietic growth factor (HGF) is an inclusive term for the family of glycoproteins that regulate proliferation and differentiation of hematopoietic cells.<sup>1-5</sup> Many of these factors also influence cell function. The term *cytokine*, a natural product of cells that influence other cells, is sometimes used as a synonym for *growth factor*. Some cytokines that function as HGFs are also called *interleukins* (ILs), regulatory factors produced by leukocytes that influence functions of other leukocytes. *Colony-stimulating factor* (CSF) is used in naming some cytokines that stimulate hematopoietic cells to grow in vitro in culture systems, such as granulocyte CSF (G-CSF), granulocyte-macrophage CSF (GM-CSF), and macrophage CSF (M-CSF). Another CSF, formerly called *multi-CSF*, is now most commonly called *IL-3*. The HGFs, their names, and principal functions are outlined in Table 32-1.

### Cell Biology and Physiology

The HGFs share some general structural features among themselves and with other cytokines, but each is the product of a different gene and has a distinctive amino acid sequence, secondary and tertiary structures, receptor-binding domain, and glycosylation sites.<sup>2,3</sup> Some HGFs, such as erythropoietin, are produced by relatively few cells at selected tissue sites (eg, the juxtaglomerular cells of the kidney). Others, such as G-CSF, are produced almost ubiquitously by endothelial cells, fibroblast cells, and many other cell types. The factors governing production and secretion also vary substantially. The feedback regulation of erythropoietin in relation to tissue oxygenation is well characterized. Although the relationships governing leukocyte production and deployment are less well understood, it is known that complete absence of G-CSF or its receptor leads to severe neutropenia.<sup>6-8</sup>

Improved immunoassay systems have greatly aided understanding of the pharmacokinetics and tissue binding of HGFs.

Neutrophilia occurs with many acute inflammatory conditions and in some malignant diseases, and is largely attributable to the increased production and levels of G-CSF.<sup>9-11</sup> Levels of G-CSF are elevated in patients with severe neutropenia who have fever and infection.<sup>10</sup> However, assay systems for G-CSF are not sensitive enough to determine whether there is a true direct feedback relationship between plasma levels of G-CSF and blood neutrophil counts. The presence of high-affinity receptors for the CSFs on both immature and mature neutrophils further confounds the study of these relationships. Levels of some other cytokines that influence neutrophil formation (eg, IL-1 and IL-6) also increase with inflammation.<sup>12</sup> In contrast, serum levels of GM-CSF, another factor that stimulates neutrophil formation, do not correlate with the presence of neutropenia or infection.<sup>10</sup> Levels of thrombopoietin (TPO), the platelet growth factor made primarily in the liver, vary inversely with platelet counts and are reduced with advanced liver disease.<sup>3,13</sup>

Proliferation, differentiation, and stimulation of hematopoietic cells occur through factor-specific cell surface receptors composed of dimers or trimers of transmembrane proteins.<sup>1-3</sup> There are general structural similarities in the extracellular and intracellular domains of receptors for many HGFs, and there are common components to the receptors for some factors (eg, a common  $\beta$  chain of the receptors for IL-3, IL-5, and GM-CSF). Specificity results from the unique, or private, components of the receptor for each factor. Receptor binding and cellular activation lead to conformational changes in the receptor, activation of intracellular kinases, and phosphorylation of specific proteins that are transferred to the cell nucleus.<sup>2</sup>

### Pharmacokinetics and Pharmacodynamics

The pharmacologic properties of HGFs depend on the dose, route of administration, affinity of binding to receptors, and complex circulatory and distribution factors, as with other drugs. There are extensive pharmacokinetic and pharmacodynamic data on erythropoietin, G-CSF, and GM-CSF (the growth factors

**Table 32-1.** Hematopoietic Growth Factors

| Factor                    | Other Name                                                                | Cell Source                                                           | Chromosome            |                                                                                                                           |
|---------------------------|---------------------------------------------------------------------------|-----------------------------------------------------------------------|-----------------------|---------------------------------------------------------------------------------------------------------------------------|
|                           |                                                                           |                                                                       | Location              | Function                                                                                                                  |
| EPO                       | Erythropoietin                                                            | Juxtaglomerular cells                                                 | 7q                    | Stimulates erythrocyte formation and release from marrow                                                                  |
| TPO                       | Thrombopoietin, MGDF                                                      | Hepatocytes, renal and endothelial cells, fibroblasts                 | 3q 27                 | Stimulates megakaryocyte proliferation and platelet formation                                                             |
| G-CSF                     | Granulocyte colony-stimulating factor; filgrastim; lenograstim            | Endothelial cells, monocytes, fibroblasts                             | 17q 11.2 q21          | Stimulates formation and function of neutrophils                                                                          |
| GM-CSF                    | Granulocyte-macrophage colony-stimulating factor                          | T lymphocytes, monocytes, fibroblasts                                 | 5q 23-31              | Stimulates formation and function of neutrophils, monocytes, and eosinophils                                              |
| M-CSF                     | Macrophage colony stimulating factor; colony-stimulating factor-1 (CSF-1) | Endothelial cells, macrophages, fibroblasts                           | 5q 33.1               | Stimulates monocyte formation and function                                                                                |
| IL-1 $\alpha$ and $\beta$ | Endogenous pyrogen<br>hemopoietin-1                                       | Monocytes, keratinocytes, endothelial cells                           | 2q 13                 | Proliferation of T, B, and other cells; induces fever and catabolism                                                      |
| IL-2                      | T-cell growth factor                                                      | T cells (CD4+, CD8+), large granular lymphocytes (NK cells)           | 4q                    | T-cell proliferation, antitumor and antimicrobial effects                                                                 |
| IL-3                      | Multicolony-stimulating factor; mast cell growth factor                   | Activated T cells; large granular lymphocytes (NK cells)              | 5q 23-31              | Proliferation of early hematopoietic cells                                                                                |
| IL-4                      | B-cell growth factor; T-cell growth factor II; mast cell growth factor II | T cells                                                               | 5q 23-31              | Proliferation of B and T cells; enhances cytotoxic activities                                                             |
| IL-5                      | Eosinophil differentiation factor; eosinophil colony-stimulating factor   | T cells                                                               | 5q 23.3-q32           | Stimulates eosinophil formation; stimulates T- and B-cell functions                                                       |
| IL-6                      | B-cell stimulatory factor 2; hepatocyte stimulatory factor                | Monocytes, tumor cells, B and T cells, fibroblasts, endothelial cells | 7p                    | Stimulates and inhibits cell growth; promotes B-cell differentiation                                                      |
| IL-7                      | Lymphopoietin 1; pro-B-cell growth factor                                 | Lymphoid tissues and cell lines                                       | 8q 12-13              | Growth factor for B and T cells                                                                                           |
| IL-11                     | Plasmacytoma stimulating activity                                         | Fibroblasts, trophoblasts, cancer cell lines                          | 19q 13.3-13.4         | Stimulates proliferation of early hematopoietic cells; induces acute phase protein synthesis                              |
| IL-12                     | Natural killer cell-stimulating factor                                    | Macrophages, B cells                                                  | 5q 31-33; 3p 12-q13.2 | Stimulates T-cell expansion and IFN- $\gamma$ production; synergistically promotes early hematopoietic cell proliferation |
| LIF                       | Leukemia inhibitory factor                                                | Monocytes and lymphocytes; stromal cells                              | 22q                   | Stimulates hematopoietic differentiation                                                                                  |
| SCF                       | Kit ligand; steel factor                                                  | Endothelial cells; hepatocytes                                        | 4q11-q20              | Stimulates proliferation of early hematopoietic cells and mast cells                                                      |

MGDF = megakaryocyte growth and development factor; IL = interleukin; NK = natural killer; SCF = stem cell factor.

approved for clinical use) but relatively little data on the other factors.

Intravenously administered recombinant erythropoietin has a circulating half-life of 4 to 13 hours in patients with chronic renal failure and approximately 20% less in healthy volunteers.<sup>14</sup> Therapeutically effective levels are maintained for at least 24 hours, after a single intravenous or subcutaneous injection. An increase in reticulocyte counts occurs within 10 days if erythropoietin is given daily. Exogenous erythropoietin accelerates erythropoiesis, but detectable increases in hematocrit and hemoglobin level usually take approximately 2 weeks to develop.<sup>14</sup> Increasing the degree of glycosylation or the attachment of polyethylene glycol to the erythropoietin molecule increases its size, delays its renal clearance, and extends the duration of its biological effects.<sup>15-16</sup> For most clinical applications, erythropoietin is administered weekly or biweekly.

Granulocyte colony-stimulating factor can be administered intravenously or subcutaneously. After intravenous administration, the elimination half-life is approximately 3.5 hours.<sup>17</sup> Blood levels are influenced by the neutrophil count, because neutrophils actively bind the injected drug.<sup>18</sup> G-CSF stimulates proliferation of neutrophil progenitors and rapidly mobilizes mature neutrophils from the marrow reserves. It increases blood neutrophils threefold to fourfold in hematologically healthy persons within 4 to 6 hours.<sup>19-20</sup> A sustained increase in blood neutrophils occurs if administration of G-CSF is continued on a daily basis.<sup>20</sup> Granulocyte colony-stimulating factor accelerates neutrophil formation, which leads to an increased percentage of circulating band neutrophils with more deeply staining primary granules. These changes are similar to those commonly seen with bacterial infection.

Granulocyte-macrophage colony-stimulating factor can be administered intravenously or subcutaneously, but intravenous administration has been associated with toxicity that includes fever, dyspnea, and acute respiratory distress syndrome.<sup>21,22</sup> These effects seem to be dose related and to occur less frequently with subcutaneous administration. Similar to G-CSF, GM-CSF stimulates neutrophil production and release of cells from the marrow. When given repeatedly for several days, GM-CSF increases total white cell count, including eosinophils and monocytes as well as neutrophils.<sup>23</sup> The leukocyte count returns to the baseline level when administration of G-CSF or GM-CSF is discontinued.

Results of in-vitro studies show that both G-CSF and GM-CSF influence the function of neutrophils.<sup>5,24,25</sup> Both types of CSF “prime” neutrophils to increase their responsiveness to other stimulators of the neutrophil oxidative (respiratory) burst, such as opsonized particles or bacteria. Neutrophils and other leukocytes produced in vivo in response to G-CSF or GM-CSF stimulation in general have normal or increased, but not decreased, responsiveness to exogenous stimulators of neutrophil function. The clinical significance of these findings is not clear. Substantial clinical data and numerous animal experiments indicate that neutrophils produced in vivo in response to these cytokines are

capable of killing bacteria and accelerating recovery from bacterial infection.<sup>5</sup> For some pathogens, in-vivo G-CSF treatment may enhance the microbicidal activity.<sup>26</sup>

The pharmacologic and physiologic properties of other HGFs are less well understood, but some generalizations are possible. The factors influencing the early phases of hematopoiesis tend to have multiple side effects, such as fever and flu-like symptoms, and a narrower range of tolerable doses than do factors that influence the later phases of hematopoiesis. Examples of early factors with substantial side effects include IL-1, IL-2, IL-3, IL-4, IL-6, and stem cell factor. The predominantly late-acting factors such as erythropoietin, G-CSF, GM-CSF, and M-CSF are much better tolerated.

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## Clinically Approved Agents and Products in Development

Erythropoietin is approved for treatment of anemia in several clinical settings: to treat anemia of chronic renal failure, anemia in chronic zidovudine-treated human immunodeficiency virus (HIV)-infected patients, anemia in cancer patients on chemotherapy, and to reduce allogeneic blood transfusions in anemic surgery patients. Erythropoietin is available as a glycosylated recombinant protein from multiple manufacturers and as an identical 165-amino acid protein (darbepoetin) that differs from recombinant human erythropoietin in containing five *N*-linked oligosaccharide chains, whereas recombinant human erythropoietin contains three. Darbepoetin has delayed clearance and a longer biological effect permitting less frequent administration; it has been widely investigated as a stimulus to erythropoiesis.<sup>27,28</sup> A pegylated erythropoietin was shown to be effective in an open-label, parallel group, noninferiority trial involving 1115 patients with the anemia of chronic renal failure.<sup>16,29</sup> Currently, the long-term safety of all forms of erythropoietin is under review. An increase in the risk of venous thrombosis has been recognized from the earliest trials; recent questions also relate to an increased risk of malignancies observed as a secondary endpoint in some clinical studies.<sup>30-32</sup>

G-CSF and GM-CSF have been approved by the United States Food and Drug Administration (FDA) for the management of neutropenia in specific clinical situations. G-CSF is indicated for cancer patients receiving myelosuppressive chemotherapy, patients with acute myeloid leukemia receiving induction or consolidation chemotherapy, cancer patients receiving marrow or progenitor cell supportive therapy, and patients with various forms of severe chronic neutropenia. A pegylated form of G-CSF (PEG G-CSF) has a much longer blood half-life and longer biological effects and can be used once per chemotherapy cycle rather than on a daily basis. Chapter 15 describes the role of G-CSF in granulocyte collection and transfusion, a use similar to that for collection of hematopoietic progenitor cells (HPCs) but not yet approved by the FDA. GM-CSF is indicated following induction chemotherapy in acute myeloid leukemia,

for mobilization of marrow progenitor cells, and to accelerate marrow engraftment after hematopoietic transplantation.

Interleukin-11 is currently approved to prevent the need for platelet transfusions following myelosuppressive chemotherapy in adult patients with nonmyeloid malignancies at risk of severe thrombocytopenia. Adverse effects including fever, edema, atrial arrhythmias, and syncope have limited its use.

Several promising new agents are in development for treatment and prevention of thrombocytopenia, but none are currently approved by the FDA. The native (TPO) molecule was first identified and cloned in 1994 by several groups almost simultaneously.<sup>33-37</sup> Its receptor (TPO-R) is expressed on megakaryocytes, platelets, and primitive HPCs.<sup>3,13</sup> Soon after this discovery, preclinical data indicated that recombinant human TPO (rHuTPO) and a truncated version of TPO coupled to polyethylene glycol called megakaryocyte growth and development factor (PEG-rHuMGDF) stimulated a twofold to fivefold increase in platelet count with a peak occurring 10 to 14 days after beginning the stimulus.<sup>38</sup> Phase I and II clinical trials demonstrated the effectiveness of PEG-rHuMGDF to raise platelet counts, but the development of neutralizing antibodies and significant thrombocytopenia in some subjects led to discontinuation of clinical trials with these specific agents.<sup>39</sup>

A recombinant TPO-R protein with a carrier Fc domain to increase its size and reduce renal clearance (currently referred to as AMG531) has recently undergone Phase I, II, and III testing and appears to be safe and effective for treatment of chronic refractory idiopathic thrombocytopenic purpura (ITP) in trials for up to 96 weeks with weekly administration.<sup>40-42</sup> In a randomized, double-blind, placebo-controlled Phase III trial involving 63 patients with ITP who had previously had splenectomies, responses with either a durable or transient increase in platelet counts were observed in 33 of 42 treated patients and 0 of 21 patients receiving placebo.<sup>42</sup> The therapy was well tolerated; no neutralizing antibodies were observed. One subject had reversible evidence of increase marrow reticulins. A venous thrombosis occurred in one patient, but this did not prevent continuation of AMG531 treatment.<sup>42</sup> This drug has been licensed by the FDA for use in Chronic ITP.

Eltrombopag is an orally active TPO-R chrome agonist shown to increase platelet counts in healthy adults, and in patients with ITP<sup>43</sup> or thrombocytopenia caused by hepatitis C infection and cirrhosis of the liver.<sup>44</sup> Eltrombopag is a small-molecule, nonpeptide oral agonist to the TPO-R.<sup>45,46</sup> The clinical trial involving 118 ITP patients with platelet counts less than 30,000/ $\mu$ L showed that 80% of patients receiving 50 or 75 mg/day responded to this therapy.<sup>43</sup> The results in the hepatitis C trial were similar; headaches were the most common adverse event in both trials. Other oral agents (eg, AKR-501 and SB-559448) are under investigation.<sup>47</sup>

Despite several clinical trials, there are no approved uses for IL-3 or M-CSF.

### Use of Myeloid Growth Factors to Mobilize Peripheral Blood Stem Cells

For years it has been recognized that circulating HPCs are capable of forming hematopoietic colonies in vitro in culture systems

and of repopulating the marrow after total ablation. These cells appear to function normally to sustain hematopoiesis and to allow expansion in states of hematopoietic stress.

Before the availability of HGFs, several investigators reported that the number of circulating HPCs increases during the recovery period after hematotoxic chemotherapy. This occurs usually after about 10 to 16 days, when marrow recovery is in progress.<sup>48,49</sup> It was subsequently learned that a similar response occurs in hematologically intact persons given HGFs after about 5 to 7 days.<sup>50,51</sup> Numerous studies have shown that the number of these progenitor cells, also called *peripheral blood stem cells* (PBSCs) or *peripheral blood progenitor cells*, predictably increases in the blood as much as 100-fold during recovery from chemotherapy or 4 to 6 days after administration of HGF is begun.<sup>52-57</sup> The quantity and quality of cells are sufficient for PBSCs autologous reconstitution after myeloablative therapy and use of PBSCs is rapidly replacing marrow for that purpose.<sup>58</sup> Peripheral blood stem cells are preferred because of the relative ease of procurement, more rapid restoration of blood counts compared with marrow infusion, and the possible recovery of these cells from patients who previously received intensive chemotherapy or radiation therapy and from patients with tumors involving the marrow.<sup>58</sup>

An unexpected benefit of the development of PBSC support came with the recognition that transfusion of PBSCs accelerated recovery of platelet counts and thus obviated the need for many platelet transfusions.<sup>53</sup> This finding suggests that the persisting thrombocytopenia historically seen 3 to 6 weeks after conventional marrow transplantation was caused by the limited number or function of precursor cells infused rather than deficiencies in humoral or environmental factors.

The basic physiologic principles governing mobilization of PBSCs probably involve breakage of the molecular bonds between key adhesive marrow elements, ie, the receptor-ligand pairs, which hold the progenitor cells in the extravascular space of the marrow juxtaposed to the marrow stromal cells. In primates, antibodies to the adherence protein VLA-4 can induce more rapid PBSC mobilization.<sup>59</sup> The chemokine IL-8 has similar effects.<sup>60</sup>

Treatment with G-CSF is recognized to expand the total myeloid and progenitor mass, increase the number of circulating blood neutrophils, and also release neutrophil-associated proteases into the marrow and blood.<sup>61,62</sup> There is good evidence that the released proteases disrupt key adhesive bonds holding the progenitors in the marrow, including the receptor-ligand pair CXCR-4 on the progenitors and stromal-cell-derived factor 1 (SDF-1) on the marrow stromal cells. The CXCR-4 antagonist AMD 3100 has similar effects in that this molecule also disrupts these bonds and promotes release of progenitor cells into the peripheral blood.<sup>63-65</sup>

All mobilizing agents cause leukocytosis with an increase in blood neutrophils as well as increased numbers of circulating HPCs, but they do not proportionately increase the numbers of circulating promyelocytes, myelocytes, or metamyelocytes. With G-CSF the kinetics of the response (delay of a few days before

the increase in PBSCs occurs) suggests that early hematopoietic cells may need to expand in numbers before they “spill over” or are “released” into the blood. The increase in PBSCs in the recovery phase from chemotherapy generally occurs when the marrow is still relatively hypocellular, thus implying that marrow cell density is not the simple explanation for mobilization.<sup>65</sup>

In the initial development of support with PBSCs, many investigators questioned whether these cells are truly hematopoietic stem cells. Most of the cells collected and transfused after treatment with HGFs or chemotherapy undoubtedly are differentiated precursor cells. However, results of experiments with mice, dogs, and nonhuman primates as well as with humans support the contention that preparations of PBSCs do contain long-lived precursor cells along with many differentiated cells.

## Technical Considerations

### Quantitation

Peripheral blood stem cells are quantified according to the cell surface expression of cluster determinant 34 (CD34) or granulocyte-macrophage colony-forming unit (CFU-GM) as measured with an in-vitro colony-forming assay. Because the in-vitro bioassay requires approximately 2 weeks to complete and is highly dependent on the expertise of the tissue culture laboratory, it is used largely for quality control and for research studies. Flow cytometric analysis for CD34+ cells can be performed quickly and easily on the day of collection and is essential to determine the adequacy of PBSC collection. Total mononuclear cell numbers also are useful. Cell numbers that usually correlate with engraftment are  $4 \times 10^8$  or more mononuclear cells per kilogram,  $2 \times 10^6$  or more CD34+ cells per kilogram, and  $2 \times 10^5$  or more CFU-GMs per kilogram. Because this antigen is expressed on cells that have differentiated beyond the colony-forming stage, the number of CD34+ cells is 10-fold greater than the required number of CFU-GMs.<sup>66</sup>

### Cytokine Doses

Currently, G-CSF is the cytokine most frequently used to mobilize PBSCs. At doses of 5 µg/kg or less per day, the harvest of PBSCs usually is inadequate. Doses of approximately 10, 16, 24, and 32 µg/kg/day have been used in clinical trials. Although the yield may be slightly increased at higher doses, the usual dose is 10 to 16 µg/kg/day.<sup>58</sup> Granulocyte-macrophage colony-stimulating factor, IL-3, and stem cell factor (*c-kit* ligand) also mobilize PBSCs, but have greater side effects. The role of thrombopoietin in mobilization is still uncertain.<sup>67</sup> Combinations of G-CSF with each of these cytokines or with chemotherapy have been investigated at many centers. In general, it seems that a regimen of chemotherapy plus G-CSF (with or without a second cytokine) yields the greatest quantity of CD34+ cells and allows for the fewest leukapheresis procedures, but there is not yet a standard approach. The timing of the response to chemotherapy is quite variable, making this approach more difficult for patients and their families. Cyclophosphamide, carboplatin, paclitaxel, etoposide, and ifosfamide are the chemotherapeutic agents that

have been studied most extensively for mobilization and harvesting of PBSCs.

## Collection and Processing of Peripheral Blood Stem Cells

Currently, PBSCs are collected and used for both autologous and allogeneic hematopoietic stem cell transplantation.<sup>53,56</sup> Under these circumstances, the type and intensity of previous chemotherapy or radiotherapy affect collection of PBSCs. Extensive previous treatment may render adequate PBSC collection difficult, if not impossible. Most collections are performed using techniques and procedures evaluated by committees of the AABB, Foundation for the Accreditation of Cellular Therapy, International Society for Cellular Therapy, and American Society for Apheresis. When patients are treated with HGFs alone, collection usually is begun on treatment Day 4. Up to 20 liters of blood are usually processed to separate predominantly mononuclear cells, and the CD34+ cell collections from the first and succeeding days are counted to determine the total number of leukapheresis procedures to be performed.

### Tumor Cell Contamination

Both marrow aspirated for transplantation and apheresis-collected PBSCs may contain tumor cells. The number of contaminating cells seems to be determined according to marrow involvement, stage of disease, tumor type, and previous therapy. Previously untreated patients tend to have greater marrow reserves or mobilizable tumor cell burdens. Techniques for detecting tumor cells in these preparations are rapidly improving. It is unclear what the minimal tumor cell contamination must be to avoid the risk of tumor cell engraftment with PBSCs mobilized by administration of HGFs.<sup>68-70</sup>

## Selection for Peripheral Blood Stem Cells

Because CD34 is a useful marker of early hematopoietic cells, several methods have been devised in which antibodies to CD34 are used for selective collection of PBSCs. The goal is to select PBSCs and eliminate tumor cells from the materials to be transfused. At present, these technologies do not eliminate all tumor cells completely but reduce the numbers markedly. Studies are still in progress to determine the clinical advantage of these procedures.

### Cryopreservation

Peripheral blood stem cells collected after treatment with HGFs usually are preserved by means of controlled rate freezing with 10% dimethylsulfoxide (DMSO). Results of some studies suggest that HGFs may inhibit spontaneous apoptosis, but it is unclear whether this is clinically important for preservation of PBSCs.

## Clinical Trials of Growth-Factor-Mobilized

### Peripheral Blood Stem Cells

Numerous uncontrolled trials and a few randomized, controlled clinical trials have shown the efficacy of HGF-mobilized PBSCs to accelerate marrow recovery after intensive chemotherapy or

myeloablative chemoradiation therapy. The results of these studies have established that administration of more than  $4 \times 10^6$  CD34+ PBSCs per kilogram mobilized by G-CSF results in neutrophil recovery to more than 500/ $\mu$ L and platelets to 20,000/ $\mu$ L in approximately 10 to 12 days.<sup>62</sup> These recovery times probably can be achieved without treating the recipient with HGFs after infusion of PBSCs. These times for recovery with PBSCs are considerably shorter than those after autologous marrow transplantation, particularly for platelets. Platelet counts recover to 20,000/ $\mu$ L by approximately Day 12 after treatment with PBSCs without G-CSF or GM-CSF therapy and to 20,000/ $\mu$ L on approximately Day 20 or later after autologous marrow transplantation.

### Alternative Agents

Although the use of HGFs to mobilize PBSCs has greatly facilitated hematopoietic transplantation, there is a need to further improve this procedure. The discovery that SDF-1 and CXCR-4 play critical roles in hematopoietic cell homing was soon followed by identification of an antagonist to the binding of this receptor-ligand pair. AMD 3100 is a small organic chemical inhibitor of this interaction that was discovered in the process of screening for agents with anti-HIV properties.<sup>64</sup> AMD 3100 is administered subcutaneously and stimulates the semiselective release of CD34+ cells from the marrow to the blood in a dose-dependent fashion.<sup>71-73</sup> Clinical studies in human volunteers have established that AMD 3100 increases CD34+ cells at 6 to 10 hours after a single subcutaneous dose sufficient for collection of greater than  $5 \times 10^6$  cells/kg by standard leukapheresis. Clinical trials in normal volunteers and patients with multiple myeloma and non-Hodgkin lymphoma have demonstrated that AMD 3100 used in combination with G-CSF is more effective than either alone.<sup>73</sup> The additive effects of AMD 3100 to G-CSF are especially important for patients who are poor mobilizers because of their underlying disease or previous chemotherapy.<sup>74</sup> FDA approval of AMD 3100 is pending, as of this writing. Other similarly acting new agents are in development.

### Other Considerations

Many laboratories have intensively studied the expansion of marrow cells and PBSCs using in-vitro incubation of the cells with a mix of growth factors, including stem cell factor, IL-3, IL-6, GM-CSF, G-CSF, and erythropoietin. This approach is a promising way to expand the number of differentiated progenitor cells for supportive care. It also may prove useful for providing undifferentiated and stem cell support if, eventually, cells can be expanded without stimulating differentiation. Paralleling these efforts, many investigators are studying genetic alteration of PBSCs mobilized by HGF administration with the intent to introduce therapeutically important genes into PBSCs. This gene therapy strategy may prove useful for the management of genetic, infectious, and malignant diseases. This work is still at an investigational stage.

### Granulocyte Colony-Stimulating Factor and Granulocyte-Macrophage Colony-Stimulating Factor for Neutropenia Associated with Chemotherapy and Hematopoietic Stem Cell Transplantation

For many years, the myelosuppressive effect of chemotherapy has been the dominant factor determining treatment doses and schedules in therapy for hematologic and nonhematologic malignant diseases. It is generally believed that recovery of blood leukocyte and platelet counts is delayed because of the time required for cell proliferation and differentiation as well as for the generation of endogenous factors regulating cell recovery. The potency of G-CSF and GM-CSF to stimulate recovery, and the effectiveness of prophylactic administration of G-CSF to stimulate recovery were easily recognized in the first clinical trials.<sup>75,76</sup> In two randomized, controlled trials,<sup>77,78</sup> the effectiveness of prophylactic administration of G-CSF was definitively demonstrated in the care of patients with small-cell lung cancer. In these studies, patients treated with cyclophosphamide, doxorubicin, and etoposide received either placebo or G-CSF at a dose of 5  $\mu$ g/kg/day. The incidence of fever and documented infections was reduced in the G-CSF-treated group both in the first cycle and for the overall six cycles of treatment. Antibiotic use and days of hospitalization also decreased. The results of this trial have been replicated in a number of studies.<sup>79</sup> Meta-analyses and comprehensive reviews have confirmed the benefit to prevent febrile neutropenia and to prevent deaths from sepsis in adults treated with G-CSF.<sup>80,81</sup> Randomized clinical trials also have shown that marrow recovery after transplantation is accelerated by GM-CSF and G-CSF.<sup>82</sup>

The results of these trials have led to a number of conclusions and practice guidelines.<sup>83-85</sup> Granulocyte colony-stimulating factor effectively stimulates marrow recovery after chemotherapy for many malignant diseases, including myeloid and nonmyeloid leukemia. It has proven to be difficult to establish whether GM-CSF is equally effective. In healthy subjects and patients, GM-CSF is less potent as a stimulus to increase neutrophil production and thus to raise blood neutrophil counts.<sup>86</sup> In addition, GM-CSF may cause fever and inflammatory symptoms, making it difficult to use the occurrence of febrile neutropenia as the primary endpoint for clinical trials.

The greatest value of CSF treatment is in the care of patients with sustained neutropenia and a high likelihood of developing febrile neutropenia and severe infections. Conversely, it has been easier to show the clinical benefit of CSF treatment in trials of single-agent chemotherapy with a lower risk of febrile neutropenia.<sup>87</sup> This apparent paradox is attributable to effects of the chemotherapy on the hematopoietic tissues. More chemotherapy causes more severe and protracted neutropenia, but it also reduces the amount of target tissue for the CSF. The net effect is that the benefits of CSF treatment, particularly to accelerate hematopoietic recovery, are greater with milder chemotherapy treatments. For this and other reasons, the American Society of Clinical Oncology and other groups have revised their original guidelines to set a 20% risk of febrile neutropenia as the level for demonstrable benefit of prophylactic CSF.<sup>83,84</sup>

In any patient with documented febrile neutropenia after one course of chemotherapy, the use of these factors to avoid

infectious complications while maintaining dose intensity is generally indicated unless a dose-reduction strategy is more appropriate because of the disease, stage of the disease, comorbidities, or personal choice of the patient. The use of G-CSF and GM-CSF in the management of myeloid malignant disease has been studied extensively. It appears that these agents can be used safely to accelerate marrow recovery after chemotherapy, as in the management of nonmyeloid malignant disease.

The timing and dosing of G-CSF or GM-CSF are largely based on schedules used in randomized clinical trials, but many questions remain to be answered. Because of the potential for the CSF to increase hematotoxicity if given before or concomitant with chemotherapy, they are generally recommended to be given the day following chemotherapy. The duration of CSF treatment is also not well established. The best evidence of their effectiveness comes from the large randomized clinical trials that proved their benefits. In these trials the CSF was given until the blood counts were normal or above normal.<sup>77-80</sup> Conversely, the treatment period may have been longer than necessary in these trials. In general it would appear that treatment should be started without delay approximately 24 hours after completion of any course of chemotherapy and continued until there are clear signs of increasing blood neutrophils before stopping this supportive therapy. Uncertainty also exists about optimal dosing. For GM-CSF, dosing may be limited by side effects. For G-CSF, the acceptable dose range is much broader, but evidence suggests that doses greater than 5 µg/kg/day do not significantly accelerate marrow recovery. The G-CSF dose of 5 µg/kg/day is the current standard of practice.<sup>83-85</sup>

### Colony-Stimulating Factors for Idiosyncratic Drug-Induced Neutropenia

A wide array of drugs induce transient neutropenia, presumably on an immune or toxic basis. The penicillins, sulfonamides, and antithyroid and psychotropic drugs often are implicated. With respect to treatment, the offending (or presumably offending) agent should be discontinued, and patients with a fever should be treated with broad-spectrum antibiotics. Numerous case studies and small series have suggested that HGFs may accelerate marrow recovery in this setting. However, the evidence is largely anecdotal, and practices differ considerably. The sickest patients with documented infections and delayed marrow recovery are the ones most likely to benefit from CSF treatment.<sup>88</sup>

### Colony-Stimulating Factors in the Management of Chronic Neutropenia

The clinical benefit of long-term G-CSF therapy for patients with severe congenital neutropenia (including Kostmann syndrome), severe idiopathic neutropenia, or cyclic neutropenia, was established in a randomized clinical trial.<sup>89</sup> In a study of patients with severe chronic neutropenia, the occurrence of fever, oropharyngeal ulcers, infections, hospitalization, and antibiotic use were all significantly reduced with dose-adjusted, daily G-CSF treatment. The quality of life and activity profiles of these patients also improved. The principal side effect of G-CSF therapy was musculoskeletal pain early in

the course of treatment, which became less severe with long-term therapy. Data from the Severe Chronic Neutropenia International Registry have shown that the subgroup of patients with severe congenital neutropenia are at risk of developing myelodysplasia and acute myeloid leukemia.<sup>90,91</sup> The risk appears to be disease specific with a substantially lower, or no risk for patients with cyclic or idiopathic neutropenia. At present, hematopoietic transplantation is the only available alternative therapy, and it is limited largely by the availability of a suitable well-matched marrow donor. For these reasons, careful follow-up evaluation of patients receiving long-term G-CSF therapy, especially patients with severe congenital neutropenia, is indicated with regular blood counts and clinical observation and annual marrow examination.

### Colony-Stimulating Factors in the Management of Nonneutropenic Infectious Disease

Considerable research efforts have been devoted to the possible use of CSFs as adjunctive therapy for nonneutropenic infectious conditions. The rationale for these studies was to enhance the number and functional activities of neutrophils to promote recovery from, or prevention of, local or systemic infections. On the basis of results of Phase II studies, a set of large Phase III trials were conducted. In a double-blind, controlled, multicenter trial, 756 patients with community-acquired bacterial pneumonia were enrolled to receive intravenous antibiotics plus either G-CSF (300 µg/day for up to 10 days; *n* = 380) or placebo (*n* = 376).<sup>92</sup> Outcome measures included time to resolution of morbidity, 28-day mortality, length of hospital stay, and occurrence of adverse events. A microbial cause of pneumonia was identified for 56% of patients, and an independent review group judged use of antimicrobial agents appropriate in the care of 98% of the patients. Administration of G-CSF increased the peripheral blood neutrophil count threefold, but the primary endpoint—time to resolution of morbidity, mortality, and length of hospitalization—was not affected. Subgroup analysis showed that the time of resolution of infiltrates was significantly more rapid in the G-CSF-treated group. Administration of G-CSF was safe and well tolerated. Further studies of more severely ill patients with pneumonia also showed similar results, with no significant effect on the primary endpoint; however, there were intriguing findings with the secondary and subgroup analyses.<sup>93</sup>

Because of a lack of neutrophilia and impaired superoxide generation in neutrophils from patients with diabetes, G-CSF appears to be a reasonable candidate for adjunctive therapy in the management of severe infections in these patients. In a small randomized, double-blind, placebo-controlled trial, 40 patients with insulin-dependent diabetes and foot infections were assigned to receive either G-CSF (*n* = 20) or placebo (*n* = 20) for 7 days.<sup>94</sup> Both groups were treated with similar antibiotic and insulin regimens. After 7 days of G-CSF treatment, there was earlier eradication of pathogens, quicker resolution of cellulitis, shorter hospital stay, and shorter duration of antibiotic treatment. Other trials, however, have not confirmed these findings.<sup>95</sup> These results await corroboration in a multicenter trial before adoption of use of G-CSF for this purpose in routine clinical practice.<sup>96</sup>

Colony-stimulating factors may have therapeutic potential in the treatment of patients with HIV. Granulocyte-macrophage colony-stimulating factor has been shown to partially correct HIV-1-mediated impairment in the ability of monocyte-derived macrophages to phagocytose *Mycobacterium avium* complex in vitro.<sup>97</sup> In a study involving 258 patients with HIV-1 infection and moderate neutropenia, G-CSF treatment significantly reduced the incidence of severe neutropenia and bacterial infection.<sup>98</sup> Patients treated with G-CSF also had 54% fewer episodes of severe bacterial infection and needed 45% fewer days of hospitalization for management of bacterial infection than did the control group. The use of G-CSF to prevent or treat infections has been suggested in numerous other small trials, but randomized, controlled trials are necessary to establish the benefits of such practices.

### Granulocyte Colony-Stimulating Factor for Mobilization of Neutrophils in Granulocyte Transfusion Therapy

Granulocyte transfusion therapy would appear to be a rational approach to the management of severe bacterial and fungal infections in patients with prolonged neutropenia. Potential clinical efficacy, however, has long been limited by insufficient donor stimulation regimens and suboptimal leukapheresis techniques. Methodologic progress, in particular for mobilization of neutrophils in healthy donors by means of administration of G-CSF and dexamethasone, has greatly enhanced leukapheresis yields. Granulocyte transfusion therapy is discussed in detail in Chapter 20.

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## Summary

The discovery and clinical development of HGFs have had great effect on the use of blood components in the supportive care of patients with many hematologic and malignant diseases. Hematopoietic growth factors are providing new opportunities for the provision of blood services, such as collection of PBSCs, autologous erythrocyte transfusion for elective surgical procedures, and G-CSF mobilization of neutrophils for granulocyte transfusion therapy.

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## Disclaimer

W. C. Liles has disclosed no conflicts of interest. D. C. Dale has disclosed financial relationships with Amgen, Genzyme, and Cellera.

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# Hematopoietic Progenitor Cells: Biology and Processing

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Hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) used for hematopoietic reconstitution after myeloablative therapy were among the first cellular therapy products shown to have a real clinical benefit. The ability of the HSC to engraft and differentiate to produce all blood cell lineages, and the ethical issues surrounding the much publicized advances in embryonic stem cell research, have both prompted experimentation into additional therapeutic uses for HSCs and HPCs. A partial list of potential clinical uses of these hematopoietic cells is presented in Table 33-1. Many have not yet been proven safe or effective.

As the field of hematopoietic transplantation has progressed, much knowledge has been gained concerning the complex interactions between cell populations contained in the donor's marrow, mobilized peripheral blood progenitor cell (PBPCs), or umbilical cord blood (UCB) graft and the recipient's cells. As a result, much has been learned in the laboratory about how to selectively deplete or enrich for subpopulations of donor cells before infusion. These cells then can serve functions other than conventional marrow replacement or rescue. They can be used for adoptive immunotherapy,<sup>1,2</sup> as vehicles for gene therapy,<sup>3</sup> and perhaps even to induce tolerance of an organ transplant from the same donor.<sup>4</sup> There are several clinical situations where such in-vitro manipulations have been shown to provide significant clinical benefit.

Transfusion medicine and clinical laboratory technology have grown to include cell processing techniques that concentrate, separate, isolate, purify, enrich, deplete, cryopreserve, and quantitate cellular components contained within marrow, peripheral blood, and UCB. The more common, clinically relevant processing techniques used to prepare hematopoietic grafts for infusion are reviewed in this chapter. The focus is on the rationale and the basic principles behind the techniques, but select technical details and literature references are also provided to assist laboratory professionals with the task of preparing standard operating

**Table 33-1.** Potential Therapeutic Uses of Hematopoietic Stem and Progenitor Cells

|                                       |
|---------------------------------------|
| Hematopoietic cell transplantation    |
| Autologous; allogeneic                |
| Myeloablative, nonmyeloablative       |
| Adoptive immunotherapy                |
| Cardiac repair                        |
| Revascularization of microcirculation |
| Repair of damaged cardiac muscle      |
| Peripheral vascular disease           |
| Bone fracture                         |
| Muscle/tendon repair                  |
| Gene therapy                          |
| Fanconi anemia                        |
| Chronic granulomatous disease         |
| Severe combined immunodeficiency      |

procedures (SOPs). This chapter concludes with a discussion of the quality assurance and regulatory compliance principles that apply to any laboratory performing cell manipulations for therapeutic purposes.

## Sources of Hematopoietic Progenitor Cells

Human HSCs and HPCs reside primarily in the marrow, but can also be obtained in clinically relevant cell doses from cytokine-mobilized peripheral blood, and UCB. These cells can also be found in other locations, such as fetal liver, but their usefulness is limited by either the low cell numbers that can be obtained or by ethical issues. A partial list of the cellular characteristics of each of these source tissues is presented in Table 33-2.

Clinical-scale marrow collections often contain a significant amount of peripheral blood, thus they contain all hematopoietic cell types, from the most immature stem cells that enable long-term engraftment to the most mature granulocytes and erythrocytes with naturally short life spans. Marrow usually

**Table 33-2.** Cellular Characteristics of Various Stem Cell Sources

|                             | <b>Marrow</b> | <b>Mobilized<br/>Peripheral Blood</b> | <b>Umbilical<br/>Cord Blood</b> |
|-----------------------------|---------------|---------------------------------------|---------------------------------|
| Stem cell content           | Adequate      | Good                                  | Low                             |
| Progenitor cell content     | Adequate      | High                                  | Low                             |
| T-cell content              | Low           | High                                  | Very low                        |
| Risk of tumor contamination | High          | Low                                   | Not applicable                  |

contains between 0.5% and 1% CD34+ cells. Optimal post-transplant hematopoietic recovery requires 2 to  $5 \times 10^6$  CD34+ cells/kg.<sup>5</sup> Therefore a clinical-scale marrow harvest should contain 2 to  $5 \times 10^8$  total nucleated cells/kg. This generally requires a 1- to 1.5-liter collection for an adult recipient. Their large volume and complex cellular makeup make most selection procedures difficult. ABO compatibility is essential, unless a red cell or plasma depletion procedure is performed before infusion.

Mobilized PBPC products are routinely used as an alternative source of HSCs and HPCs for transplantation.<sup>6-8</sup> The CD34+ repopulating cells can be mobilized into the peripheral blood and then collected by apheresis following myelosuppressive chemotherapy and/or stimulation with 10 to 20 µg/kg granulocyte colony-stimulating factor (G-CSF).<sup>9,10</sup> Because an apheresis procedure is used to collect PBPC products, they contain very few erythrocytes or granulocytes, compared to marrow, and are primarily composed of mononuclear cells (MNCs). PBPC products also contain larger numbers of HPCs than either marrow or UCB, and therefore facilitate faster engraftment and shorter hospital stays. Patients who have been heavily pretreated with multiple rounds of chemotherapy or radiation therapy often mobilize poorly and require multiple collection episodes.<sup>11</sup>

PBPC products contain larger numbers of T cells than marrow collections, and thus present a greater risk of causing graft-versus-host disease (GVHD) in the allogeneic setting, although the rate of acute GVHD is less than originally feared.<sup>12-16</sup> Mobilized peripheral blood is easier to collect than marrow and, therefore, presents a lower risk to the volunteer donor. However, the long-term risks of G-CSF administration remain unclear.

The availability and relative ease of collection of cord blood stem cells, along with the reported decreased rate of GVHD, makes UCB transplantation an attractive option. Because of the limited number of HPCs that can be acquired from this source, however, most recipients have been children.<sup>17-19</sup> UCB contains a high proportion of HSCs and HPCs, but only approximately 10% of the cell dose of a typical marrow harvest; therefore, engraftment is often delayed with UCB transplants relative to marrow or PBPC transplants.<sup>20-22</sup> Some centers have begun to test whether infusing 2 UCB units would improve engraftment kinetics.<sup>23</sup> It appears that either the number or the alloreactivity of the T cells are reduced in UCB grafts, enabling the use of partially mismatched unrelated donors with relatively low rates of GVHD.<sup>24-26</sup> With its high proliferative

potential, UCB may be a good target for gene therapy,<sup>27</sup> and/or ex-vivo expansion.<sup>28</sup> However, results of studies of the ability of ex-vivo expanded portions of UCB grafts to shorten the time to engraftment have generally been disappointing.<sup>29-32</sup>

Even though each of these tissue sources contain HPCs capable of long-term engraftment, the products vary dramatically in terms of red cell content, granulocyte and platelet content, T-cell number, and CD34 number. Thus, they present different processing objectives and challenges to the processing laboratory. No two HPC products received for processing are the same, in terms of donor age, disease status, tumor cell infiltration, infectious disease status, or prior chemotherapy exposure. Therefore, processing results (purity, recovery, viability) will often vary for unknown reasons.

There are distinct advantages and disadvantages to using a particular HPC source tissue for transplantation. The decision regarding which source to use for a particular patient is best left to the patient's physician so that disease status and donor availability can also be factored into the decision. The preferred, sought-after donors of allogeneic HPCs have customarily been sibling donors who are HLA identical at the HLA-A, -B, and -DR loci. When such a match cannot be found, related donors mismatched at one or two HLA loci are used, as are HLA-matched unrelated volunteer donors from the international registries.

## Collection of Progenitor Cells

### Marrow Harvests

Marrow harvests are invasive surgical procedures performed in an operating room under general anesthesia. Approximately 1000 to 1500 mL of marrow is collected, 3 to 10 mL at a time, from multiple punctures of both posterior iliac crests, using specially designed stainless steel beveled needles and syringes. The marrow is collected into a disposable collection apparatus that contains a 500-micron filter and a 200-micron filter to remove most of the bone chips, clots, fat, and fibrin before the marrow is drained into a collection bag that contains an anticoagulant. Target cell doses often range between 2 and  $5 \times 10^8$  nucleated cells/kg and between 2 and  $5 \times 10^6$  CD34+ cells/kg. Collection protocols vary, but most often the syringes are heparinized in advance with 1 mL of a 100 U/mL heparin solution. The marrow is drained into a collection bag containing the same heparin solution, such that the heparin concentration is at least 10 U/mL in the final product. Additional heparin may be required if there is to be a long-distance transport or overnight hold of the marrow before processing. Although the marrow donor usually remains in the hospital overnight, it is possible to perform the marrow harvest as an outpatient procedure with local anesthesia. Complications from the general anesthesia or from infection at the anatomic site of the harvest can occur.

### Peripheral Blood Collections via Apheresis

With the development of recombinant colony stimulating factor (CSFs), it has become possible to mobilize large numbers of

**Table 33-3.** Instrument Settings for Peripheral Blood Progenitor Cell Collections

| Instrument | RBC Content (%) | Flow Rate (mL/min) | Anticoagulant to Whole Blood Ratio | Cycle Volume (mL) | Number of Cycles |
|------------|-----------------|--------------------|------------------------------------|-------------------|------------------|
| Spectra    | 1 to 3          | 60 to 150          | 1:12 to 1:15                       | Continuous        | N/A              |
| Amicus     | 6 to 8          | 40 to 75           | 1:12 to 1:15                       | 1000 to 1400      | 7 to 14          |
| COM.TEC    | 6 to 8          | 40 to 60           | 1:10 to 1:14                       | 300 to 500        | 25 to 40         |

progenitor cells into the peripheral circulation so that they can be collected by a much less invasive procedure. By stimulating the donor with either hematopoietic growth factors, or chemotherapy and growth factors, a sufficient number of circulating stem cells for marrow rescue can be collected in one to three apheresis procedures.<sup>10</sup> Although the peripheral blood of healthy individuals contains fewer than 0.1% HSCs, this number increases dramatically during recovery from cytotoxic therapy and even more so when recombinant CSFs such as G-CSF are administered. Peak counts usually are obtained 5 days after stimulation with G-CSF (10 to 20 µg/kg/day) or 10 to 14 days after chemotherapy and G-CSF. Daily monitoring of peripheral blood CD34 counts is useful for determining when to start leukapheresis after chemotherapy and G-CSF mobilization. Most centers begin leukapheresis when the peripheral CD34 count reaches or exceeds 10 CD34+ cells/µL. Stem cell factor, thrombopoietin, and flt-3 ligand have also been reported to successfully mobilize HPCs.<sup>33-35</sup>

Peripheral blood stem cells can be used as a source of stem cells for both autologous and allogeneic marrow transplants. A PBPC collection is performed with a cell separation device originally developed for therapeutic plasmapheresis, leukapheresis, and platelet donation procedures. This apheresis device uses a centrifuge to separate and collect MNCs, including peripheral HSCs, from the blood. In order to achieve a target cell dose of 2 to 5 × 10<sup>6</sup> CD34+ cells/kg, it is necessary to process 12 to 25 liters of blood or 2.5 to 6.0 times the patient's calculated blood volume. Investigators have reported that the yield of CD34+ cells increases continuously as more blood volumes are processed.<sup>36</sup> Although up to six times the donor's blood volume can be safely processed, some donors are not able to tolerate 4 or 6 hours being connected to an apheresis machine. Therefore, these donors require repeated collections on sequential days once the peripheral CD34 count has increased to acceptable levels (>10 CD34+ cells/µL) for collection.

There are currently three different commercially available apheresis instruments. In each case, instrument settings such as inlet flow rate, centrifuge speed, collect pump flow rate, and anticoagulant:whole blood ratio vary, depending on the target cell type to be collected. The three instruments operate differently. The Amicus (Baxter, Deerfield, IL) and the COM.TEC (Fresenius SE, Bad Homburg, Germany) are more automated, computer-controlled instruments; however, the Spectra (CaridianBCT, Lakewood, CO) is more widely used. Suggested settings for the collection of CD34+ progenitor cells using these instruments are presented in Table 33-3. Note that the red cell content setting for the Spectra is set with a colorgram. Targeting a colorgram of 1% to 3% results in a product with approximately 6% red cells,

whereas this parameter can be entered directly into the other two instruments. Also, because the final product is more concentrated when collected by the Amicus, adding 100 to 150 mL of autologous plasma is recommended to rinse the collection line and to improve viability during storage.

A high red cell content in PBPC collections can cause problems with patient/donor ABO incompatibility in allogeneic transplants, and cause processing issues when performing cell selection procedures. The CD34+ (MNC) layer is just below the platelet layer. Going deeper into the white cell layer (closer to the red cell layer) increases the collection of granulocytes (PMNs), which can cause increased clumping and clotting in the products. In order to achieve the desired product hematocrit, the collect valve may be closed until the desired interface is established.

Two donor-related issues require special consideration. First is the issue of priming of the apheresis machine. Even when devices to that minimize extracorporeal volume are used, priming of the apheresis machine with red cells is required for individuals in whom the volume of the tubing set exceeds 10% to 15% of the total blood volume. This step prevents unacceptable dilutional anemia during the procedure and prevents fluid overload associated with the return of red cells from the centrifuge chamber at the end of the procedure. Second is the use of acid-citrate-dextrose (ACD) anticoagulant. The citrate component of ACD poses a risk of symptomatic hypocalcemia, which can limit the rate of blood processing and/or the duration of the procedure. Calcium replacement should be strongly considered to prevent adverse events during the apheresis collection procedure.

The anticoagulant:whole blood ratio is important to both the product and the donor. Increasing the ratio can reduce the collection time, but it also reduces the amount of anticoagulant being mixed with the donor's whole blood and thus increases the potential for clotting inside the tubing set and/or the product bag. When making adjustments to this ratio, one must consider the donor's platelet count from the day of collection.

### Umbilical/Placental Cord Blood Collection

Harvesting of UCB is the least invasive of all HSC collection techniques. The cord is clamped, and a 16-gauge needle attached to a standard blood donor set containing a preservative, usually ACD formula A (ACD-A) or citrate-phosphate-dextrose-adenine (CPDA-1), is inserted into the umbilical vein. Cord blood collection can either be performed by the obstetrician with the placenta in situ, or immediately after delivery by a trained team that can process the placenta in a location in close proximity to the delivery room. If cells are collected after delivery, the placenta

can be suspended from a specially constructed frame to aid in drainage of the cord blood. The usual volume collected is less than 170 mL.<sup>37</sup> After collection by gravity, some additional blood remaining in the placenta can be expressed by means of physical massage of the organ. A device that applies pressure to the placenta in a funnel-like framework has been reported to provide increased yields in total volume and CD34+ content.<sup>38</sup>

## Processing Procedures

A few general principles must be followed to preserve product integrity and to protect the operator from harm. Collection of a good quality unit is the ultimate goal.

- *Practice universal precautions.* All products are potentially infectious and if handled carelessly, can spread disease to the operator, or to another product. Donors should be tested for transfusion-transmitted diseases in advance of the procedure.
- *Follow aseptic processing guidelines.* Closed systems are of course preferable, but if not available, adherence to strict aseptic processing guidelines, such as use of a biosafety cabinet, is essential to protect the product from environmental contaminants.
- *Prepare.* Time is a critical factor with these products; however, it is important to perform the procedure in a deliberate manner. Preparation, including taking time to prepare materials, reagents, workspace, worksheets, and labels in advance of product arrival frequently reveals problems that can be solved in advance, thereby improving product quality.
- *Follow the SOP.* The SOPs serve as a step-by-step guide during processing. These procedures need to be written clearly, kept in a convenient location, updated easily, version-controlled, and consulted frequently during processing to prevent errors—even if the procedure is being performed by experienced personnel. Assuming the procedures have been validated to result in the desired endpoint, they must be followed as written.
- *Observe.* Although it is important to follow the SOP as written, it is also important to be alert for product abnormalities or unexpected outcomes that might necessitate a procedural deviation.
- *Document.* The benefits of having detailed documentation on file for each procedure performed cannot be overstated. Such documentation is needed for accreditation by voluntary standard-setting organizations [AABB, Foundation for the Accreditation of Cellular Therapy (FACT), College of American Pathologists (CAP), The Joint Commission], regulatory review by federal agencies [Clinical Laboratory Improvement Act (CLIA) and Food and Drug Administration (FDA)], and for best practice analyses during any internal or external quality systems/outcome reviews.

As new techniques are developed, or existing procedures modified, the specific aims will of course vary. However, there are two common goals of all in-vitro manipulations of HPCs. First, there must be minimal loss of, or damage to, the progenitor cells. The operator needs to ensure that the cells are collected and processed in accordance with the existing standard of care, so

that an adequate number of viable cells will remain after processing to provide for robust engraftment in a reasonable amount of time. Second, the processing technique must not place the component at undue risk of contamination with microorganisms. All procedures must be performed under aseptic conditions, preferably in a closed system, with appropriate assays for detection and identification of infectious agents at critical steps. Several of the more commonly used cell processing procedures are listed in Table 33-4, and described below.

### Red Cell Depletion

When the donor and recipient have a major ABO incompatibility (patient has antibodies to donor red cells), infusing more than 20 mL of incompatible red cells can cause a serious hemolytic transfusion reaction. Often, red cells are removed from marrow and UCB products to debulk the product before cryopreservation or as a prerequisite to subsequent processing. In the latter case, the absolute residual red cell content is less important, so a simple buffy coat preparation may suffice. Red cell depletion of PBPC products is rarely necessary because the apheresis procedure results in relatively low level red cell content.

Red cells can be depleted from marrow or UCB products by use of several different techniques. Historically, red cell depletion strategies were either based on density differences between cell types,<sup>39</sup> or a differential sensitivity to hypotonic solutions. More recently, hetastarch has been used to cross-link red cells such that they rapidly settle out of solution. Exposure of red cells to a 1:5 v:v ratio of 6% hetastarch results in the formation of cross-links between multiple red cells, significantly increasing their weight and causing them to rapidly settle to the bottom of a V-bottom container (bag).<sup>40</sup> Within 45 to 60 minutes, the leukocyte-rich plasma layer can be expressed into a separate collection bag or the red cell pellet can be drained into a waste container. Some marrow contains an excessive number of red cells; thus normalizing the hematocrit before adding the hetastarch can be beneficial. In the authors' experience, this method results in a good leukocyte recovery (>90%) with approximately 95% reduction in red cell content, resulting in <20 mL residual red cells. This maneuver allows for safer product transfusion to an ABO-incompatible recipient. A somewhat less pure product results from centrifugation followed by removal of the leukocyte-rich buffy coat layer.

### Plasma Depletion

Plasma depletions are also commonly performed on allogeneic PBPC and marrow products to prevent donor antibodies to recipient ABO antigens from causing a transfusion reaction following product infusion or to simply reduce the volume of the product in order to prevent volume overload. The product is divided into multiple 300-mL or 600-mL transfer packs and centrifuged at approximately  $900 \times g$  for 10 minutes. The plasma is extracted, and the infranatant fluids are combined into one container. The addition of a heparinized isotonic buffer solution containing a small amount of protein (human serum albumin, HSA) helps preserve cell viability and improves the infusion flow rate.

**Table 33-4.** Commonly Used Cell Processing Procedures

| Aim                        | Rationale                                                                                                                                                                 | Methods                                                                                                                                                                                                                |
|----------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Volume reduction           | Ease of storage and infusion                                                                                                                                              | Centrifugation                                                                                                                                                                                                         |
| Plasma depletion           | Reduce volume of ABO-incompatible plasma                                                                                                                                  | Centrifugation                                                                                                                                                                                                         |
| Red cell depletion         | <ul style="list-style-type: none"> <li>• Reduce number of ABO incompatible red cells</li> <li>• Debulk product</li> </ul>                                                 | <ul style="list-style-type: none"> <li>• Hetastarch sedimentation</li> <li>• Ficoll-hypaque</li> <li>• Buffy coat <math>\text{NH}_4\text{Cl}</math> lysis</li> </ul>                                                   |
| T-cell depletion           | <ul style="list-style-type: none"> <li>• Reduce GVHD in allogeneic recipients</li> <li>• Eliminate autoreactive T cells from autografts (autoimmune disorders)</li> </ul> | <ul style="list-style-type: none"> <li>• Centrifugal elutriation</li> <li>• Antibody + complement lysis</li> <li>• Solid-phase monoclonal antibodies</li> <li>• Stem cell selection</li> <li>• Immunotoxins</li> </ul> |
| Malignant cell depletion   | Eliminate tumor from autografts                                                                                                                                           | <ul style="list-style-type: none"> <li>• Antibody + complement lysis</li> <li>• Stem cell selection</li> <li>• Immunotoxins</li> <li>• Cell culture</li> </ul>                                                         |
| Stem cell enrichment       | <ul style="list-style-type: none"> <li>• Reduce tumor burden</li> <li>• Reduce GVHD</li> <li>• Facilitate expansion/gene marking</li> </ul>                               | Stem cell selection                                                                                                                                                                                                    |
| Donor lymphocyte infusions | <ul style="list-style-type: none"> <li>• Reduce graft rejection</li> <li>• Increase donor chimerism</li> <li>• Facilitate GVL response</li> </ul>                         | CD3 aliquots                                                                                                                                                                                                           |
| Cryopreservation           | HPC storage                                                                                                                                                               | Controlled-rate freezing with cryoprotectants                                                                                                                                                                          |
| Gene transfer              | <ul style="list-style-type: none"> <li>• Correct defective HPC</li> <li>• Confer drug resistance</li> </ul>                                                               | <ul style="list-style-type: none"> <li>• Retroviral transduction</li> <li>• Lentiviral transduction</li> </ul>                                                                                                         |

GVHD = graft-vs-host disease; GVL = graft-vs-leukemia; HPC = hematopoietic progenitor cell

One must be careful, however, that infusion volumes don't exceed approximately 20 mL/kg.

### Mononuclear Cell Enrichment

Marrow and UCB (and to a lesser extent PBPCs) are heterogeneous collections of cellular and noncellular components. The cells that lead to hematopoietic reconstitution are mononuclear; other cell populations in the graft may be superfluous, or cause harm. For example, most of the cells in a marrow or UCB product are mature red cells and granulocytes, which lyse and release potentially harmful enzymes during freezing and thawing. The CS3000 (Baxter) and the Spectra (CaridianBCT) use an automated differential centrifugation technique to remove approximately 95% of the red cells from a marrow product, while approximately 75% of the MNCs are recovered in a final product that contains more than 80% MNCs.<sup>41</sup>

### CD34 Selection

Immature progenitor cells possess a surface marker (CD34) that gradually disappears as the hematopoietic cells mature and

differentiate.<sup>42,43</sup> CD34 selection is used for the purpose of tumor purging, T-cell depletion, or to provide a starting population for gene therapy or stem cell expansion procedures. Two different CD34 selection devices are approved for clinical use in the United States or Europe. Both positively select antibody-coated CD34+ cells by immunomagnetic reactions that allow the CD34+ cells to be retained in the device while the antigen-negative cells are eluted. However, the details of the selection methods differ.

The Isolex 300i System (Baxter Oncology, Deerfield, IL) is the only CD34 selection device currently approved by the FDA for use in autologous PBPC transplantation. This system is almost fully automated and consists of four steps: 1) labeling cells with an anti-CD34 monoclonal antibody (MoAb); 2) mixing sensitized cells with paramagnetic beads coated with sheep anti-mouse IgG, whereby CD34+ cells form rosettes with the beads; 3) attaching the rosettes to a magnet and washing CD34- cells from the system; and 4) releasing the cells from the beads using a peptide mimotope (a synthetic polypeptide designed to mimic the CD34 antigen) that competes with the MoAb binding site of the CD34 molecule.<sup>44-46</sup> The magnetic field retains the beads and residual

antibody/peptide complexes in the separation chamber while the CD34+ cells are collected in a separate bag. All washes (including the initial wash to remove platelets) are performed within the closed system apparatus by using a unique spinning membrane technology. Reagent additions, incubations times, cell washing, and other steps in the selection are all software controlled. Depending on the protocol, the selected cells are then prepared either for immediate infusion, cryopreservation, or further manipulation.

The CliniMACS system is based on the immunomagnetic cell selection technology developed by Miltenyi Biotec.<sup>47,48</sup> This device is approved for clinical CD34+ cell selection in Europe, Canada, and some other countries and is currently undergoing clinical trials in the United States. In this partially automated system, the PBPC or red-cell-depleted marrow product is washed manually to remove platelets and plasma, and is then treated with a murine anti-CD34 that is conjugated to a stable colloidal suspension of super-paramagnetic particles composed of iron oxide and dextran. The cells are placed into the disposable tubing set in the selection device and the CD34+ cells are selected on a high-gradient magnetic separation column. The magnetically labeled cells are held in the column while unbound cells are washed through. The magnetic field is removed and the selected cells are collected. Buffer additions and flow rates are controlled via a computer-controlled system of peristaltic pumps, fluid sensors, and pinch valves. The retained antibody/particle complex does not affect the functional capacity of the selected cells. Other antibody-antigen systems can be employed for clinical application if performed under the appropriate FDA-approved investigational new drug (IND) protocol.

CD34 selection is considerably more efficient when starting with PBPC apheresis products than it is with either marrow or UCB, or frozen/thawed products. It is possible to routinely achieve greater than 90% CD34 purity and greater than 70% CD34 recovery when selecting CD34+ cells from PBPC, but recovery and purity both suffer when selecting CD34+ cells from either marrow or UCB. Preselection stem cell enrichment techniques such as density centrifugation or red cell depletion are helpful for achieving a better purity and recovery. Adding a small amount of a clinical grade DNase and magnesium chloride to older products delayed in transit or frozen/thawed products, greatly reduces clumping and improves postselection purity and recovery. Even with fresh PBPC, the starting product quality can affect selection performance. Products with low hematocrits and low granulocyte content are preferable, as are products with low to normal platelet counts.<sup>49</sup>

### T-Cell Depletion

The process of T-cell depletion (TCD) removes potentially host-reactive immunocompetent T cells from an allogeneic graft, resulting in a dramatic reduction in the incidence of GVHD. Unfortunately, if too many T cells are removed, this process may also increase the incidence of graft failure and leukemia relapse, suggesting that some residual T cells are beneficial in an allogeneic product. Rigorous depletion of 4 logs also significantly delays T-cell immune reconstitution, especially CD4+

T cells, after transplantation. Conversely, if not enough T cells are removed, GVHD may occur, particularly if the donor and recipient are not fully HLA-matched. Some centers prefer to use the most rigorous depletion procedure available (immunomagnetic, as discussed below), and then add back defined doses of T cells or T-cell subsets to combat graft failure or speed immune reconstitution. Other centers have chosen to do a less complete TCD and use additional posttransplant immune suppression to prevent GVHD. Thus, the choice of TCD procedure to employ should be based on the desired target dose for infusion. The critical number of T cells needed to support the graft-vs-leukemia effect has not yet been determined. However, transplantation with a T-cell dose of  $1 \times 10^5/\text{kg}$  body weight allows engraftment and minimizes the severity of GVHD. TCD may also be used to remove autoreactive T cells from autografts used to treat patients with various severe autoimmune diseases. This approach appears promising but is currently considered to be experimental.

Early T-cell depletion techniques were based on the phenomenon by which T cells form rosettes around sheep red cells.<sup>50</sup> After rosetting, the T cells can be removed from the suspension by means of density gradient centrifugation. This technique is tedious and labor intensive, but surprisingly effective. More recently, most centers that perform T-cell depletions utilize MoAbs to T-cell antigens to remove these cells from allogeneic grafts. Methods have been developed in which antibodies to antigens such as CD3, CD4, CD5, CD6, and CD8 are used in conjunction with complement, immunotoxins, or magnetic microspheres to remove T cells from allogeneic grafts.<sup>51-53</sup> Not all TCD methods target the total CD3+ T-cell population. Depleting only the CD8+ subset of CD3+ T cells may achieve similar outcomes to total TCD without as many of the negative side effects. Some centers even target only those donor T cells that respond to the recipient alloantigens.<sup>54</sup> Performing a CD34 selection also produces an infusion product with 4 log fewer T cells than were contained in the original product.<sup>55-57</sup>

### Donor Lymphocytes

Donor T cells that are reactive to recipient minor histocompatibility antigens can cause a graft-vs-leukemia response and thereby reduce relapse rates.<sup>58,59</sup> Donor leukocyte infusion (DLI), has been used to obtain complete remissions in relapsed acute myeloid leukemia, acute lymphocytic leukemia, multiple myeloma, non-Hodgkin lymphoma, myelodysplastic syndrome, and chronic myeloid leukemia.<sup>60-62</sup> It is most successful with chronic myelogenous leukemia. Approximately 65% of patients achieve complete remission in comparison with 25% of patients with acute myelogenous leukemia or myelodysplastic syndrome.<sup>63</sup> Donor lymphocyte infusions have been most effective when administered early in the relapse. The risks associated with DLI are GVHD and marrow suppression. The risk is minimized if treatment begins with a small dose that is increased if there is no response.

Allogeneic T lymphocytes are now commonly cryopreserved either before, or as part of, the HPC collection, especially in the unrelated donor setting. The donor CD3+ T cells contained in



marrow, mobilized PBPC products, nonmobilized PBPC products, or small-volume PBPC donations are enumerated and can be given to the recipient fresh, in specified doses, or else cryopreserved in CD3+ dose aliquots. Typically, the T-cell doses infused range from  $1 \times 10^6/\text{kg}$  to  $5 \times 10^7/\text{kg}$ . Physicians typically request that multiple doses be cryopreserved for later use, in case of relapse. The same cryopreservation procedure used for HPC products is most commonly used for these small-volume products.

### Cryopreservation

Transplantation of autologous marrow and PBPC and allogeneic UCB requires a means of efficient preservation of the progenitor cells, sometimes for months or even years before transplantation. As cryopreservation techniques have improved, more allogeneic products are being cryopreserved for donor convenience, or to reduce the risk of a collection failure or transport error while a patient is immunocompromised.

Cryopreservation is among the most critical manipulations performed in clinical cell processing laboratories. Techniques vary slightly among institutions; however, in all cases, the objective is to adjust the volume to the container of choice, add a cryoprotectant solution and then freeze the cells at a controlled rate, in a manner that preserves cell viability and proliferative potential after thawing. It is essential that the cryopreservation procedure prevent ice crystal formation, prevent the formation of toxic solute concentrations that can result from cell dehydration, and stabilize the cell membrane in order to prevent damage during the thawing process. Mononuclear cells tolerate freezing relatively well, so minor procedural deviations are not usually detrimental. Even so, process variables such as volume: surface area ratio and the rate that the cryoprotectant is added should be well controlled, and monitored regularly. Many centers prefer to perform a buffy-coat procedure to reduce the red cell content in UCB and marrow products before cryopreserving them.<sup>37</sup>

There are numerous cryoprotectant solutions being used with good results. All contain a colligative agent such as dimethylsulfoxide (DMSO); one or more polymeric agents such as HSA, autologous plasma, or hetastarch; an anticoagulant; and an isotonic diluent or culture media. The standard freezing mixture contains 20% DMSO (final concentration, 10%), ACD-A (final concentration 10%), an isotonic electrolyte solution, and a source of protein, usually autologous plasma or HSA, although several laboratories prefer a lower DMSO concentration.<sup>64-67</sup>

DMSO is toxic to cells if exposed for long periods at room temperature; thus, it is important to keep the cell suspension and all reagents chilled before and after the addition of the DMSO.<sup>68</sup> In addition, diluting DMSO in aqueous solutions is an exothermic reaction. Therefore, it is important to dilute the DMSO with an aqueous buffer and refrigerate the solution before adding it to the cells to minimize the DMSO toxicity. The addition of the DMSO solution to the cells needs to occur slowly for similar reasons. Product handling procedures that occur after DMSO addition need to be gentle and need to avoid exposing the cell membranes to excessive shear forces.

Stiff et al<sup>69</sup> were the first to incorporate hydroxyethyl starch (HES) as an additional cryoprotectant. The addition of HES to the cryopreservation media allows for the reduction of the DMSO concentration, thereby decreasing the unpleasant side effects and adverse events occasionally experienced during or after DMSO infusion.<sup>70,71</sup> However, it is important to be aware of possible complications, such as hypertension, that can result from infusing large volumes of HES. A 2.5% to 6% HES concentration in combination with 5% DMSO is an effective cryoprotectant for cryopreserving hematopoietic cells.<sup>72,73</sup>

Whichever cryoprotectant is used, the freezing rate is also important for viable cell recovery. A controlled freezing rate of  $-1^\circ\text{C}$  to  $-2^\circ\text{C}$  per minute is described as optimal in most reports. Thus, the most effective freezing profiles use a liquid nitrogen freezer that is programmed to maintain a constant freezing rate of between  $-1^\circ\text{C}$  and  $-2^\circ\text{C}$ , despite the heat of fusion that occurs as the solution begins to solidify.<sup>74</sup> Slightly different profiles are required for different container sizes. A typical freezing profile is shown in Fig 33-1. The program that the freezer used to generate this freezing profile is presented in Table 33-5. After the cells reach  $-90^\circ\text{C}$ , they are transferred to a liquid nitrogen storage freezer and are stored in the vapor phase, at  $\leq -160^\circ\text{C}$ .

Some centers do not use a programmable freezer, but instead place the products into a  $-80^\circ\text{C}$  mechanical freezer for 18 to 24 hours before transferring them to liquid nitrogen storage. There are several reports in the literature claiming equivalence to controlled-rate freezing if the packaging configuration is correct.<sup>66,75-79</sup> Although the storage of HSCs at  $-80^\circ\text{C}$  does not seem to cause obvious damage during short periods, there is evidence that complete cessation of enzymatic and metabolic activity does not occur until a temperature of at least  $-135^\circ\text{C}$  has been reached.<sup>69</sup>

Once a particular method has been validated to result in rapid engraftment and an acceptable postthaw viability (using 7-amino-actinomycin D), it is important to keep variables such as product volume, container size, and maximum cell concentration consistent so that the temperature distribution is uniform.<sup>80</sup> The optimal cell concentration is still not clear, so many laboratories choose to allow this parameter to vary, up to a maximum of between 1 and  $3 \times 10^8$  cells/mL, so that the product volume per bag can be consistent from patient to patient.<sup>81</sup>

### Storage

When the freezing program is completed, the bags can be removed from the chamber and placed in prechilled and labeled protective canisters for storage. The storage temperature should be low enough to slow the metabolism and block all the enzymatic pathways; thus, the lower the storage temperature the better. Mechanical freezers can achieve temperatures as low as  $-120^\circ\text{C}$ ; however, liquid nitrogen freezers are much more reliable and provide even lower temperatures. Vapor phase liquid nitrogen storage ( $-160^\circ\text{C}$ ) has the advantage of preventing product cross-contamination and minimizes occasional bag breakage during thawing. Vapor phase storage is most frequently

**Figure 33-1.** Typical freezing curve from the cryopreservation of a PBPC product in a controlled-rate freezer. This curve was generated using the freezing profile shown in Table 33-5.

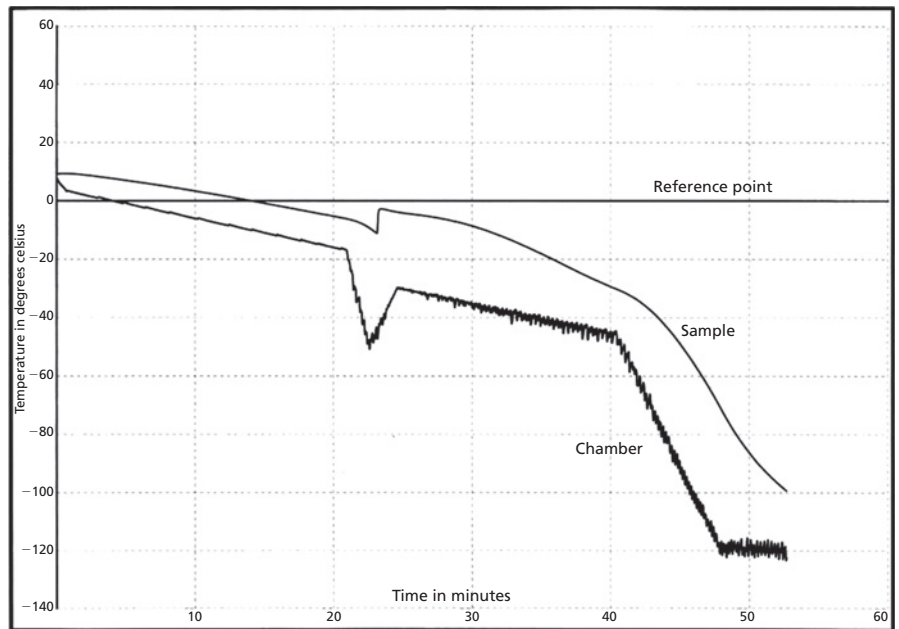


**Table 33-5.** Typical Profile for Hematopoietic Cell Cryopreservation

| Profile   | Step No. | Rate<br>(degrees<br>per min.) | End Temp.<br>(degrees C) | Trigger      |
|-----------|----------|-------------------------------|--------------------------|--------------|
| 70-mL bag | 1        | Hold                          | 4                        | Chamber temp |
|           | 2        | -1                            | -8                       | Sample temp  |
|           | 3        | -25                           | -60                      | Chamber temp |
|           | 4        | +10                           | -30                      | Chamber temp |
|           | 5        | -1                            | -30                      | Sample temp  |
|           | 6        | -10                           | -90                      | Sample temp  |
|           | 7        | Hold                          | -90                      | Chamber temp |

used for short-term (<1 year) storage.<sup>82,83</sup> Liquid phase storage ( $-196^{\circ}\text{C}$ ) is less prone to occasional temperature fluctuations as freezers are accessed, and provides a larger safety margin if the liquid nitrogen source is depleted; thus, it is frequently used for long-term storage. In either case, continuous temperature monitoring, remote alarm notification, and automatic fill features are essential to maximize product safety during storage. Overwrap bags provide an extra layer of protection against product cross-contamination, and are advisable for high-risk products.

Short-term storage of products before or after processing is best accomplished by providing nutrients, buffering the pH, and augmenting the anticoagulant solution, if appropriate. Viability is best maintained if the cells are stored at a cool temperature in a gas-permeable bag. Because it is not clear how long HPC products remain effective when stored under these conditions, it is best to minimize storage times whenever possible.

## Transportation

Product transport is essentially short-term product storage under duress, yet it is a critical maneuver for those centers that process tissues from distant clinical sites. Even the best planned courier systems fail in bad weather, or when air travel is involved, requiring last-minute adjustments in staffing schedules. Such situations have the potential to compromise the product if delays are excessive. Diluting the cells with autologous plasma and a buffer solution at the collection site, adding extra heparin for long-distance transport, and shipping at a cool temperature in a well-insulated container help significantly to protect the product. Adding a source of DNase upon arrival in the laboratory for products more than 24 hours old can also improve processing results by eliminating the free DNA released from cells that have died during transport.

All product shipment procedures need to comply with current biohazard product packaging and labeling regulations. Fresh HPC products need to be packaged in sealed, puncture-proof containers. Transportation of frozen HPC products requires the use of a liquid nitrogen dry shipper that contains absorbent material in the wall of the device to absorb the liquid nitrogen and eliminate the risk of spillage. These devices are designed to hold temperatures below  $-160^{\circ}\text{C}$  for 8 to 10 days, but the liquid nitrogen evaporation rate needs to be checked before each use.

## Thawing/Infusion

Cryopreserved cells are less prone to injury when thawed rapidly. The bags of cell suspension are thawed just outside the patient's room in sterile overwrap bags in a  $37^{\circ}\text{C}$  waterbath. The bags are thawed one at a time and immediately, but slowly, infused through a central venous catheter or intravenous access, as soon as the product reaches approximately  $15^{\circ}\text{C}$ . It is generally best if

laboratory personnel perform the thawing procedure and clinical personnel perform the infusion.

The bag of cells may be hung and allowed to drip through an administration set, or the cell suspension can be withdrawn from the bag with a syringe and injected directly into the catheter port. A few centers wash the cell suspension to reduce the volume or to remove DMSO before infusion, particularly for pediatric patients or for patients with renal insufficiency. Because of the fragility of the cells after thawing, any procedure must treat the cells gently and not subject them to vigorous shaking or centrifugation, which can cause the suspension to clump, resulting in poor cell recovery. Slowly diluting the cell suspension with approximately 1:5 cold dextran and allowing the cell membranes to stabilize before centrifugation improves viability and recovery. If any postthaw processing is anticipated, it is advisable to consider the addition of DNase during either freezing or thawing to reduce the clumping caused by single-stranded DNA from the cells that did not survive the freeze-thaw process. Platelets and plasma proteins also can cause clumping of progenitor cells after thawing.

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## Quality Control

Quality control testing plans need to be protocol-specific, and designed to demonstrate postprocessing product integrity and product safety. Product safety is the primary concern, especially for Phase I clinical trial products. However, demonstration of product integrity (potency) is also important for interpreting clinical findings.

## Safety

The tests most often performed in order to demonstrate safety are donor infectious disease testing, bacterial and fungal product sterility testing, endotoxin testing, and mycoplasma testing (for cultured products). Additional safety tests are often required by the FDA for complex products—for example, testing for the presence of replication-competent retrovirus in the final product is required when using a retroviral vector to genetically alter an HPC product.

Products need to be tested for sterility at receipt and at the end of processing. Final product testing is mandated; testing at receipt provides a way to determine whether any of the techniques or materials used have compromised the purity of the product. Even if every effort is made to maintain sterility during collection and processing of an HPC product, there is a risk of contamination with microorganisms, especially when multiple manipulations are performed. Contamination can result from microorganisms in the skin or bloodstream of the patient or donor, improperly sterilized equipment or materials, environmental contaminants, or faulty handling of the graft.<sup>84,85</sup>

Sterility testing should be performed with a validated method for detecting bacterial (aerobic and anaerobic cultures) and fungal contamination. Procedures need to be in place whereby the

patient's physician is promptly notified if a sterility test result is positive. For products released before even preliminary results of sterility testing, it is often difficult to obtain proof of product safety before infusion. It is still important to perform the cultures and to document product safety after the fact. Same-day procedures such as Gram's staining and endotoxin assays can also be helpful.

## Integrity

Integrity testing provides the patient's physician with important product purity and potency information and can be valuable for evaluating process improvements and deviations.

The most widely used potency assay for progenitor cell components is the number of viable CD34+ cells (or their subsets) present at the end of the procedure. Numerous reports in the literature have concluded that the time to hematopoietic recovery after transplantation is optimal with CD34 doses of at least  $5 \times 10^6$  per kg.<sup>86-88</sup> The total nucleated cell dose is obtained using an automated hematology analyzer. The percentage of MNCs can be determined by performing a differential count on a stained smear. Cell viability and phenotypic characterization (CD45, CD34, CD3) are best accomplished using flow cytometry.<sup>89-93</sup> Functional assays such as colony-forming ability, long-term culture-initiating ability, or animal model in-vivo hematopoietic reconstitution assays are essential for establishing the therapeutic potential of new products, but are impractical for everyday use.<sup>94</sup>

## Release Criteria

Minimal acceptance criteria for nucleated cell count, viability (FDA recommends >70%), target cell content, progenitor cell content, etc, should be established in advance. If a product does not meet release criteria, it must be further processed (to bring it into compliance), discarded, supplemented with additional product, or released with the approval of appropriate laboratory and clinical personnel.

In principle, product release criteria should have clinical relevance. With cell therapy products, however, the products are so extremely valuable to their intended recipients that physicians almost always want to infuse the cells even if they do not meet predefined acceptance criteria. Often, it is not possible to discard a contaminated product because the patient will not survive the myeloablative therapy without receiving the product. The patient's physician is notified when a product does not meet expectations and the procedures used to produce the product are thoroughly investigated.

Product release decisions should be based on objective criteria that are more encompassing than just the review of final product test results. Most release criteria are safety oriented; therefore, proper procedural documentation, raw material testing, and defined acceptance criteria for in-process test results all contribute to user confidence in product quality and should be incorporated into the quality assurance plan. The best way to design relevant release criteria is to determine under what circumstances the product should not be infused. What if the product wasn't properly labeled? What if it were known to contain human immunodeficiency virus? What if the purity was

so low that the chance of achieving the goal of the trial is too remote to justify the risks of proceeding? Such issues need to be addressed among the transplant team members.

## Quality Assurance

### Accreditation

Voluntary standards have been developed by two professional organizations in an effort to improve product quality. The AABB and FACT have published standards for the collection, processing, storage, and infusion of cell therapy products.<sup>95,96</sup> Both professional organizations offer on-site accreditation of laboratories acting in accordance with the published standards. Both systems emphasize the prevention of errors and an ongoing process of continuous quality improvement. FACT and AABB accreditation are theoretically voluntary, but are essentially mandatory because most clinical transplant programs are required to be accredited to obtain insurance contracts. Adherence to voluntary standards such as those promoted by these organizations is an excellent yardstick for predicting product performance. Accreditation should be mandated for every cell processing facility.

### Federal Regulations

All cell therapy products, including hematopoietic stem cells, are biologic products that are currently subject to regulation by the FDA via the *Code of Federal Regulations (CFR)* under 21 CFR part 1271 or 21 CFR part 211, depending on the product type. All cell processing groups must now be registered with the FDA and must be prepared for ad hoc FDA inspections. Minimally manipulated PBPC and marrow products for homologous use are regulated via FDA's current good tissue practice (cGTP) regulations that are contained in 21 CFR part 1271. The more complex biologic products, such as cord blood expansion products and gene therapy products, are regulated via FDA's current good manufacturing practice (cGMP) regulations that are contained in 21 CFR part 211.

### Quality Systems

The best way to meet all the various standards and regulations is to design and write a quality assurance plan that contains clear instructions for how to accomplish each of the universal quality assurance principles listed in Table 33-6.

Multiple resources are available for writing a quality management plan, including both FACT and AABB standards. In general, quality management plans that focus on error prevention (not error reporting) are the most effective. Many problems can be avoided by strict attention to process control. Control of the product collection, processing, transport, and storage procedures is critical. Standard operating procedures should be written clearly and concisely so that critical control points in the procedure can be monitored. Materials management procedures prevent problems by ensuring that all reagents and supplies are properly acquired, stored, and used. Validation programs are excellent tools for proving proper equipment operation and process performance. A good

**Table 33-6.** Quality Plan Essentials

|                                    |                                  |
|------------------------------------|----------------------------------|
| <b>Organization and Leadership</b> | <b>Monitoring and Assessment</b> |
| <b>Process Control</b>             | Audits                           |
| Standard operating procedures      | QC testing                       |
| <b>Materials Management</b>        | <b>Process Improvement</b>       |
| Supplier qualification             | Deviation management             |
| <b>Human Resources</b>             | Corrective and preventive action |
| Personnel training                 | <b>Equipment</b>                 |
| Staff competency                   | Validation                       |
| <b>Documents and Records</b>       | Calibration                      |
| Document control                   | Maintenance                      |
| Label control                      | <b>Information Management</b>    |
| Worksheets                         | <b>Nonconforming Events</b>      |
| <b>Facilities</b>                  | <b>Customer Focus</b>            |
| Environmental control              | Communication                    |
| Environmental monitoring           |                                  |
| Safety                             |                                  |

document control system ensures consistent SOP conformance and prevents most labeling errors. Environmental monitoring and control systems help prevent accidental contamination errors. Proper deviation reporting and corrective action tracking greatly facilitate continuous process improvement. Perhaps most important, a program of personnel training and ongoing competency assessment prevents the operator errors that are caused by miscommunication or misunderstanding. Once these systems are in place, it can be advantageous to file a type V drug master file with the FDA that can be referenced with each subsequent IND submission to speed the approval process.

## Disclaimer

T. Leemhuis has disclosed no conflicts of interest. R. Sacher has disclosed professional relationships with Talecris Biotherapeutics and GlaxoSmithKline.

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# 34

## Hematopoietic Progenitor Cells: Autologous Transplantation

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The number of patients undergoing hematopoietic stem cell (HSC) transplantation has grown rapidly as the efficacy of this treatment continues to be demonstrated in clinical trials. Transplant registries have collected data on over 65,000 transplant procedures, but this is believed to represent only a portion of all such procedures performed.<sup>1</sup> In Europe alone, over 12,000 marrow or peripheral blood stem cell (PBSC) transplant procedures are performed each year.<sup>2</sup> Much of this increase can be attributed to the adoption of PBSC products as the primary source of HSCs for transplantation, a change driven by the more rapid engraftment parameters of PBSCs, with resulting lower regimen-related risks of morbidity and mortality, and lower costs. Also, the use of high-dose chemo- and radiotherapy with stem cell rescue has increased because of success in randomized trials of dose-intensive therapy in achieving durable remissions in common dose-responsive malignancies such as multiple myeloma,<sup>3</sup> Hodgkin disease,<sup>4</sup> and non-Hodgkin lymphoma (NHL).<sup>5</sup> It has been 50 years since the discovery that cellular elements of the marrow protect against lethal irradiation.<sup>6</sup> Transplantation science during this interval is notable for the development of antineoplastic conditioning regimens achieving effective tumor cell kill, immunomodulation regimens allowing allogeneic transplantation without inevitable fatal graft-vs-host reactions, and simple HSC collection and cryopreservation techniques. An understanding of the hematopoietic and immunologic systems has continued to develop, so that enriched populations of HSCs or immunologic effector cells may be isolated and manipulated *ex vivo* to generate “somatic cell therapy” products that differ in function from native marrow cells.

Historically, HSC transplantation was developed as a technique to deliver high doses of chemotherapy with or without radiation therapy. The collection, storage, and infusion of autologous HSCs was intended to alleviate the iatrogenic marrow failure of the high-dose chemo- and radiotherapy regimen used

to treat the patient. The infusion of autologous HSCs, in contrast to allogeneic transplantation, conveys no direct immune antineoplastic benefit to the recipient given that there is no immunologic graft-vs-disease response. Autologous transplantation is, therefore, based on two considerations:

- The dose sensitivity of the disease being treated and the availability of dose-intensive regimens capable of ablating this disease.
- The ability to collect and successfully store adequate numbers of HSCs to allow rapid reconstitution of marrow function.

Although autologous HSC transplantation does not convey the immunologic graft-vs-disease effect achieved with allogeneic transplantation, the corresponding lack of graft-vs-host disease (GVHD) and the avoidance of toxic immunosuppressive medications required after allogeneic transplantation allows the use of dose-intensive therapy for older patients, including patients in their eighth or ninth decade of life, as well as for patients with comorbid conditions that might preclude allogeneic transplantation.

Both the dose and combination of agents used for chemotherapy are important in achieving cure of malignant diseases. Malignant diseases such as Hodgkin disease, intermediate-grade NHL, and acute leukemia are potentially curable if multiple chemotherapy agents are administered in a regimen that allows the use of an effective dose of each agent. The importance of combining multiple chemotherapy agents is illustrated by the increase in cure rates for Hodgkin disease treated with effective drugs in combination, in contrast to similar drugs used singly or in sequence. The importance of dose is illustrated by the lack of further increase in the proportion of patients cured by the inclusion of additional chemotherapeutic agents to a regimen, if such addition requires a reduction of dose to avoid regimen-related toxicities. Thus, the combination of mechlorethamine, vincristine, prednisone, and procarbazine (MOPP), and doxorubicin, bleomycin, vinblastine, and dacarbazine (ABVD) in alternating schedules that doubles the number of drugs is no more effective than either regimen used alone in the treatment of Hodgkin disease. This is because in combining these regimens, the dose of the individual drugs is reduced by 50% because of



the alternate-cycle administration of each regimen. Frei et al<sup>7</sup> proposed that effectiveness of a chemotherapy regimen can be estimated by calculating the “summation dose intensity” that includes the amount of drug delivered over a period of time.

The curative treatment of dose-responsive malignancies such as breast cancer is limited by the lower activity of the chemotherapy agents available and because almost all of the agents are myelosuppressive, requiring dose reduction to accommodate combination chemotherapy. However, even for breast cancer, the importance of dose density (amount of chemotherapy administered over a unit of time) was demonstrated in 1981 by Bonadonna and Valagussa<sup>8</sup> in a retrospective analysis of two of their clinical trials with cyclophosphamide, methotrexate, and 5-fluorouracil (5-FU). They found that disease-free survival (DFS) was longer for those patients who received a higher percentage of the intended dose of chemotherapy. This could reflect either the larger amount of drug delivered or that women who tolerated the drugs were also more likely to live longer. Similarly, Kummel et al<sup>9</sup> reported a randomized trial of dose-dense therapy in the adjuvant treatment of patients with node-positive breast cancers and found improved disease-free survival and overall survival in the patient group receiving the dose-dense regimen. Wood et al<sup>10</sup> showed in a prospective randomized trial that the delivery of higher doses resulted in a significantly better survival of women being treated for early breast cancer. This larger amount of chemotherapy delivered was better regardless if it was delivered in higher doses with fewer cycles or if an equivalent amount of chemotherapy was delivered in lower doses but over a longer period. The authors conclude that these data are consistent with either a dose-response effect or a threshold level of the dose or dose intensity. The importance of dose in the treatment of lung cancer and NHL is also known.

The infusion of HSCs allows the administration of a dose-intense (higher doses of drugs over a short period) regimen without concern for the effects on hematopoiesis (myeloablative regimens). The importance of dose intensity instead of dose density for many diseases is demonstrated by randomized trials of autologous transplantation for diseases such as multiple myeloma,<sup>3</sup> Hodgkin disease,<sup>4</sup> and NHL.<sup>5</sup> These trials illustrate that dose-intense therapy is more effective than available non-transplant regimens delivered in multiple cycles over time. The delivery of even more chemotherapy using sequential (tandem) transplants has been explored in a small number of clinical trials,<sup>11</sup> but careful selection of regimens is required to avoid the nonmyeloid toxicities of the regimens used.

The infusion of HSCs has also been demonstrated to facilitate the administration of dose-dense nonmyeloablative regimens, potentially achieving a doubling of the amount of chemotherapy administered.<sup>12,13</sup> The potential of this approach was most clearly illustrated by Petengell et al,<sup>13</sup> who treated 25 patients with small-cell lung cancer with multiple cycles of ifosfamide, carboplatin, and etoposide (ICE) chemotherapy. Peripheral blood stem cells were collected after each cycle of chemotherapy and used for the support of the following cycle. The investigators

were able to double the dose-density, although only about 50% of the patients were able to complete the planned therapy. Five patients reverted to standard-dose therapy at a median of chemotherapy cycle four. Three patients died while on study (two of sepsis). Others reverted to standard-dose therapy because of prolonged cytopenias (two patients) and inability to collect cells or intolerance of dimethylsulfoxide (DMSO) (one patient each). One group treated 18 patients with sarcoma with high-dose ifosfamide and doxorubicin using a single PBSC collection split into multiple fractions and cryopreserved.<sup>12</sup> The group administered a median of  $0.9 \times 10^6$  CD34+ cells/kg with each cycle of therapy; the patients experienced a granulocyte count  $<500/\mu\text{L}$  for a median of 4 days and a platelet count  $<20,000/\mu\text{L}$  for a median of 2 days. The acceptance of cell support for these dose-dense regimens is limited by the logistics of delivering cryopreserved cells in hospitals and offices without easy access to this technology.

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## Techniques of Autologous HSC Transplantation

### Patient Eligibility for Autologous HSC Transplantation

Careful patient selection and timing of treatment are critical to the success of dose-intense therapy. Patients with bulky tumor masses and patients with chemotherapy-resistant malignancies are much less likely to achieve durable control of malignancy even with the use of dose-intense regimens. The early introduction of dose-intense therapy in the treatment of a patient, before chemotherapy resistance and excessive chemotherapy-induced organ toxicity develop, is paramount. Therefore, treatment algorithms incorporating HSC salvage strategies must be part of the initial therapeutic decisions for each patient with a disease possibly treatable by high-dose therapy. Patients being prepared for autologous transplantation will frequently undergo several cycles of chemotherapy intended to:

- Demonstrate the sensitivity of the disease to chemotherapy.
- Reduce the bulk of the disease before autologous transplantation.
- Facilitate the collection of PBSCs.

Involved-field radiotherapy can be used to debulk large tumor masses but is, in general, reserved for administration after recovery from transplantation because of the deleterious effects of this treatment modality on the collection of PBSCs and the increase in organ toxicity within the treatment field with subsequent administration of chemotherapy. Radiotherapy used as consolidation therapy after autologous transplantation sometimes requires the infusion of additional HSCs to offset the marrow-suppressive effects of this therapy. This illustrates the need for comprehensive planning to ensure that reserve quantities of HSCs are available if needed.

Patients undergo a detailed evaluation to uncover any organ dysfunction that could preclude the safe administration of this therapy. Although HSC reinfusion rescues the patient from the hematologic toxicity of the conditioning regimen,

the nonhematologic (organ) toxicity of dose-intensive therapies remains. A retrospective review of 383 consecutive transplant procedures noted factors predictive of early transplant-related mortality including an FEV<sub>1</sub> (forced expiratory volume in 1 second) less than 78% of predicted, serum creatinine greater than 1.1 mg/dL, and serum bilirubin greater than 1.1 mg/dL.<sup>14</sup>

Current transplantation techniques using PBSCs minimize the period of neutropenia and the infection risk. Mortality rates reported after autologous HSC transplantation are frequently less than 5%, although this risk increases somewhat for older patients.<sup>15</sup> Because of this risk, patients undertaking dose-intensive therapy, if possible, should be free of infection and have other comorbid conditions such as diabetes mellitus under optimal medical control.

### Selection of Conditioning Regimen

Autologous HSC transplantation facilitates disease cure through the administration of dose-intensive regimens. Therefore, the conditioning regimen used should include drugs that are effective in the treatment of the particular malignancy and induce minimal nonhematopoietic organ damage at the myeloablative doses used for transplantation. Immunosuppression is not required for engraftment of autologous HSCs; therefore, agents such as antithymocyte globulin or total body irradiation (TBI) are not necessary. Some regimens, such as high-dose melphalan for the treatment of patients with multiple myeloma and carmustine (BCNU) based regimens for the treatment of lymphoma, are commonly used based on the results of multicenter Phase III studies showing the tolerability of these regimens. Relapse is the major cause of failure of autologous HSC transplantation. Therefore, some transplant programs are exploring novel techniques to deliver yet more intensive conditioning to the regimen. One example of this is the addition of radiolabeled

antibodies to a chemotherapy or TBI-based regimen to specifically target areas of tumor while sparing radiation-sensitive organs such as the liver, lung, and kidney.<sup>16</sup> In the study, 21 to 27 Gy of iodine-131-labeled anti-CD20 was added to a conditioning regimen of etoposide (20 mg/kg) and cyclophosphamide (100 mg/kg). This resulted in a 77% complete response rate and a 68% 2-year probability of progression-free survival (PFS) for patients with relapsed NHL. The 2-year PFS was almost double that previously experienced (36%) by similar patients treated with chemotherapy alone. This technique is currently being evaluated in a multicenter study sponsored by the National Heart, Lung, and Blood Institute, National Cancer Institute, and National Institute of Allergy and Infectious Diseases through the Bone Marrow Transplant/Clinical Trials Network.

### Selection of HSC Source

Peripheral blood stem cells have virtually replaced marrow as the HSC component for autologous transplantation and have shown benefit in Phase III studies in allogeneic transplantation.<sup>17</sup> Data published from a European transplant registry showed that the vast majority of autologous and all allogeneic HSC transplants were still marrow transplants in 1991.<sup>18</sup> By 2003, PBSCs were used in 97% of autologous HSC transplant procedures. The ease of collection and the rapid engraftment kinetics of PBSCs compared to marrow are widely recognized. With PBSCs, median times to achieve an absolute neutrophil count (ANC) >500/ $\mu$ L are typically about 10 to 12 days, and platelet recovery is even faster (Table 34-1).<sup>19-23</sup> In one Phase III study of 47 patients treated for germ cell tumor, rapid engraftment kinetics were achieved with PBSC transplantation. A small number of the PBSC recipients achieved a neutrophil count of >500/ $\mu$ L 1 day faster, and a sustained platelet count of >20,000/ $\mu$ L 7 days faster than the patients receiving marrow with all patients receiving

**Table 34-1.** Relationship Between Mobilization Scheme, Dose of CFU-GM or CD34+ Cells Infused, and Engraftment Kinetics After Autologous PBSC Transplantation\*

| Study                     | Patients | Therapy                | Progenitor Cell Dose              |                                  | Engraftment Kinetics (in days) |                               |
|---------------------------|----------|------------------------|-----------------------------------|----------------------------------|--------------------------------|-------------------------------|
|                           |          |                        | CFU-GM<br>$\times 10^4/\text{kg}$ | CD34+<br>$\times 10^6/\text{kg}$ | ANC<br>>500/ $\mu$ L           | Platelets<br>>20,000/ $\mu$ L |
| Nademanee <sup>20</sup>   | 39       | G-CSF                  | ND                                | 6.2                              | 10 (7-40)                      | 15.5 (7-63)                   |
| Sheridan <sup>21,22</sup> | 29       | G-CSF                  | 21.0                              | ND                               | 6 (4-10)                       | 11 (9-136)                    |
| Weaver <sup>23</sup>      | 692      | Chemotherapy†<br>G-CSF | 30.8‡                             | 9.9‡                             | 9 (5-38)                       | 9 (4-53+)                     |
| Bensinger <sup>19</sup>   | 124      | Chemotherapy†<br>G-CSF | ND                                | 9.4                              | 11 (4-20)                      | 10 (6-65)                     |

\* Shown are mean values for progenitor cell quantities infused and median values for time to achieve the particular endpoint of engraftment.

† CFU-GM cultures performed on thawed cells.

‡ Shown are median values.

CFU-GM = colony-forming unit-granulocyte-macrophage; PBSC = peripheral blood stem cell; ANC = absolute neutrophil count; ND = no data; G-CSF = granulocyte colony-stimulating factor.

granulocyte colony-stimulating factor (G-CSF).<sup>24</sup> A similar trial for patients with NHL or Hodgkin disease reported faster platelet recovery (time to a platelet count  $>20,000/\mu\text{L}$ ) of 16 vs 23 days, and faster granulocyte recovery (time to a neutrophil count  $>500/\mu\text{L}$ ) of 11 vs 14 days for recipients of PBSCs compared to recipients of marrow.<sup>25</sup> The PBSC recipients also spent fewer days in the hospital and required fewer red cell and platelet transfusions.

There are disadvantages to the use of PBSC components as a source of HSCs for autologous transplantation. These include the usual need for multiple days of collection, the current need for sophisticated flow cytometric analysis of the components to ensure adequacy of HSC content, the inability to collect adequate components from all patients, the (minimal) risks associated with administration of hematopoietic cytokines and the apheresis procedures, and the risks of infusion if the multiple components are cryopreserved. Marrow collection and infusion is available to patients for whom adequate PBSC component(s), defined by the number of CD34+ cells collected, cannot be harvested and stored.

The dose of CD34+ cells in the PBSC component(s) required for infusion depends upon the intended treatment regimen. Lower doses of CD34+ cells appear satisfactory for nonablative regimens.<sup>12,13</sup> For example, one group infused  $0.9 \times 10^6$  CD34+ cells per cycle for patients treated with high-dose ifosfamide and doxorubicin.<sup>12</sup> Despite an overall doubling of the ifosfamide dose in this Phase I study, the median times between cycles did not progressively increase, indicating that adequate marrow stores were maintained. For myeloablative regimens, increasingly higher CD34+ doses result in greater likelihood of rapid recovery of PBSC counts.

Patients who receive a dose of CD34+ cells above an ill-defined threshold experience engraftment. At lower doses of CD34+ cells there is considerable heterogeneity in engraftment speed, especially for platelet recovery, with some patients experiencing quick engraftment despite low doses of PBSCs. It is not known why this heterogeneity exists but it may reflect host marrow stromal cell damage from previous disease infiltration or chemotherapy, a weakness in the correlation between CD34+ cells and the cells responsible for hematologic recovery or, more simply, a greater degree of error in the measurement of CD34+ cells at the lower cell concentrations found for “poor mobilizers.” As the dose of CD34+ cells increases, the engraftment speed becomes more consistent for the population studied, although the median days of cytopenia and the minimum number of days is not affected.<sup>19,23</sup> Several investigators showed more consistently rapid granulocyte and platelet engraftment for recipients of products containing a quantity of CD34+ cells above a dose of about  $2$  to  $3 \times 10^6/\text{kg}$  of recipient weight.<sup>26-28</sup>

Many of the randomized studies showing an advantage of autologous HSC transplantation over other (nontransplant) therapies used marrow as the source of HSCs. The duration of aplasia predicts the incidence of peritransplant mortality after autologous transplantation.<sup>29</sup> Although this may reflect patient

characteristics rather than graft characteristics, it is possible that use of PBSCs will result in yet a greater difference in the outcome of these therapies.

### Expansion of Inadequate HSC Components

Hematopoietic stem cells are characterized by the ability to self-renew and to differentiate to mature blood cells. Collections of HSCs from the marrow or peripheral blood are termed “stem cells” despite the fact that they contain distinct populations of true HSCs and more differentiated progenitor cells with limited proliferative capacity. In-vitro techniques to increase (expand) the numbers of HSCs in components that contain limited or inadequate numbers of HSCs are being developed with the hope that expansion of HSCs will allow simpler collection techniques or the administration of dose-intense therapy to patients from whom adequate marrow or PBSC components cannot be collected. These techniques have been used in Phase I studies involving marrow, PBSCs, or umbilical cord blood.<sup>30</sup> The results of these studies are encouraging, but clinical utility has not been conclusively demonstrated and requires further study.

### Tumor Cell Purging

Hematopoietic stem cell components are a complex mixture of cells, and many of these cell populations affect the outcome of allogeneic or autologous transplantation. The preponderance of evidence demonstrates that malignant cells contained in an HSC graft are capable of causing disease after transplantation. The transmission of malignant cells in an organ graft has been demonstrated in both HSC and solid organ (eg, orthotopic heart, liver, or kidney) transplantation from living or cadaveric donors suffering from occult disease at the time the graft was harvested.<sup>31,32</sup> These reports conclusively demonstrate that even very small quantities of malignant cells can engraft when infused into a properly conditioned host. It is not surprising, therefore, that relapse of disease occurs after autologous HSC transplantation for diseases involving or likely to involve the blood or marrow.

Tumor cell contamination or minimal residual disease (MRD) of the component contributing to relapse after autologous HSC transplantation was demonstrated in studies involving the transplantation of genetically marked marrow cells from patients with acute or chronic leukemia or neuroblastoma.<sup>33-35</sup> The level of marked cells detected after relapse was very low, and these studies cannot conclude that the cell inoculum was the sole source of malignant cells causing the disease relapse. Nor do these studies demonstrate that purging of tumor cells is necessary or effective. However, the implication of these studies, as with the solid organ transplant experience, is that the infusion of tumor-contaminated HSCs may be detrimental to the recipient and purging, if without undue toxicity and of proven efficacy, may be beneficial to the subset of patients whose HSC components are contaminated by tumor cells. The unanswered question, therefore, is not whether the cell inoculum can be a source of posttransplant relapse but, rather, whether manipulation of the HSC component can affect this outcome.

It is generally accepted that collections of hematopoietic progenitor cells from the peripheral blood likely contain fewer malignant cells than marrow collections. However, circulating malignant cells have been found for patients with neuroblastoma, breast cancer, and lymphoma.<sup>36</sup> Despite the evidence cited above about the potential contribution of MRD to poor transplant outcome, no study to date in any malignant disease demonstrates a benefit from purging of HSC components intended for autologous transplantation. The hypothesis that purging is beneficial for patients with B-cell malignancies such as chronic lymphocytic leukemia (CLL) or NHL is supported by clinical reports. The University of Nebraska program reported that 10 of 11 patients who received marrow from which lymphoma cells could be cultured relapsed after transplantation compared with 2 of 13 patients who received “culture-negative” grafts.<sup>37</sup> This group subsequently reported a fourfold greater PFS of 57% vs 17%, for recipients of grafts with no detectable MRD.<sup>38</sup> Only the presence of a positive culture from the HSC inoculum predicting for a lower event-free survival (EFS) was confirmed in a subsequent randomized study conducted by this group comparing PBSCs and marrow as sources of HSCs.<sup>39</sup>

The Dana-Farber transplant program reported similar differences in DFS based on the presence or absence of detectable MRD in trials involving antibody-mediated purging.<sup>40-42</sup> These studies used sensitive polymerase chain reaction (PCR) techniques to monitor the presence of tumor cells before and after purging with monoclonal antibodies and complement. Patients who had documented persistence of tumor cells had a significantly lower probability of DFS than patients who received components that were PCR negative after purging. The difference in DFS remains highly significant even after 10 years of follow-up (Kaplan-Meier estimate of DFS probability of 80% vs 10%).<sup>43</sup> These publications provide the strongest evidence to date that complete tumor cell purging (based on the most sensitive tumor cell detection assays available) is both necessary and beneficial. This group reported much less success in achieving PCR negativity in the purging of components from patients with mantle cell NHL and a concomitant high relapse rate after transplantation for that disease.<sup>44</sup> Although the number of patients transplanted for CLL was too small for definitive conclusions, the Dana-Farber group likewise suggested a higher probability of DFS for those patients who received PCR-negative HSC components.<sup>45</sup>

These MRD detection techniques are surrogate markers for transplant outcome. It is, therefore, not surprising that other transplant programs have published contradictory reports. For example, one group reported relapses for three of four patients treated for follicular NHL whose marrow components became PCR negative after purging with one antibody and complement but for only 11 of 25 patients who received PCR-positive marrow.<sup>46</sup> A multivariate analysis by other investigators to determine the risk of relapse for 24 patients with poor-prognosis NHL who received unpurged PBSCs did not demonstrate any effect from tumor cell contamination on the risk of relapse after transplantation.<sup>47</sup> Yet, these investigators did report that the

persistence or return of tumor cells detected by molecular analysis into the blood or marrow after transplantation was predictive of relapse in 81% of the patients, and that patients with detectable disease after transplantation experienced a 24-fold higher risk of relapse compared with patients who became and remained PCR negative.

An alternative explanation for the Nebraska and Dana-Farber reports is that the detection of tumor in the graft with or without purging reflects the quantity of tumor cells in the component which, in turn, is proportional to the amount or the aggressiveness of the disease in the patient. Patients with higher body burdens of tumor are less likely to achieve a durable remission after HSC transplantation. Sharp et al,<sup>38</sup> however, assigned patients with marrow involvement to PBSC transplantation, so the better PFS observed by them after PBSC transplantation does not support the contention that marrow contamination reflects only overall body burden of tumor. Although many patients relapse at sites of previous disease, this could reflect preferential trafficking into previous sites of disease of malignant lymphocytes reintroduced with the HSC inoculum.<sup>48</sup> Recurrence at previous sites of disease is not conclusive evidence that residual disease in the patient is the only source of relapse after autologous HSC transplantation. The discrepancy between the experiences of these various centers could reflect the importance of adequate purging, differences in patient selection, or the efficacy of the conditioning regimen. This discrepancy also illustrates the need for properly designed studies to directly address the benefit and toxicity of purging. A variety of purging agents have been tested in Phase I and II studies. The numbers of patients and financial resources estimated to be required for definitive Phase III tests of purging efficacy have discouraged the conduct of these studies.

### Cryopreservation of HSC Components

The primary difficulty in using HSC components to reduce the hematologic toxicity of dose-intense regimens is the necessity for cryopreservation. Three considerations apply. First, current cryopreservation techniques for PBSC components require cryopreservation of both the HSCs that account for about 1% of the nucleated cell population of the component and the mature blood cells that do not contribute to the recovery of marrow function after infusion. These mature cells both increase the volume of the component (thereby increasing the amount of cryoprotectant solution added) and appear to contribute to the postinfusion morbidity experienced by the recipient.<sup>49</sup> Second, the recipient of cryopreserved HSC components may experience considerable toxicity from the DMSO used in the cryoprotectant solution. Third, the cryopreservation of HSCs is technically difficult. Long-term cryopreservation of cells using DMSO requires storage and shipping of cells at a temperature below  $-135^{\circ}\text{C}$ , generally using liquid nitrogen containers. The thawing of large numbers of bags of cells is usually performed by a skilled laboratory technologist at the patient's bedside.

A high incidence of generally mild, infusion-related morbidity with the reinfusion of cryopreserved cells has been reported by

several centers.<sup>50-52</sup> Dimethylsulfoxide has a variety of pharmacologic effects.<sup>53</sup> The LD<sub>50</sub> values (amount of DMSO required to kill 50% of test animals) reported for intravenous infusion of DMSO are 3.1 to 9.2 g/kg for mice and 2.5 g/kg for dogs.<sup>54</sup> The acute toxic dose of DMSO for humans has not been determined. If a large amount of cryopreserved material is to be infused (eg, >1 g DMSO/kg of patient weight), the infusion can be separated over 2 days to avoid complications from infusion of excessive amounts of DMSO. Because the osmolality of thawed marrow (with 10% DMSO) is about 1800 mOsm/kg, central venous catheters are the preferred route of administration. The incidence of the more common reactions appears to be related to the volume of the product infused.

The most dramatic toxicity is the rare anaphylactic reaction occurring during the initial administration of thawed cells. This appears to be an allergic reaction to DMSO or other components of the solution used for cryopreservation and, after resuscitation of the recipient, the remainder of the cells should be administered cautiously. Nonallergic, profound hypotension can result from the intravenous infusion of DMSO, presumably from histamine-induced vasodilation, especially for patients who were not adequately premedicated with appropriate antihistaminic medications such as diphenhydramine. Skin flushing, dyspnea, abdominal cramping, nausea, and diarrhea, reported to varying degrees after HSC infusion, can also be attributed to DMSO-induced histamine release. These complaints resolve over a few hours and should be treated symptomatically.

Dimethylsulfoxide affects the cardiovascular system in a variety of ways. In a series of 82 patients premedicated with diphenhydramine, both increased blood pressure and decreased heart rate, which became maximal about 1 hour after the completion of the marrow infusion, were observed.<sup>50</sup> Cardiac arrest or high-degree heart block occurring during or immediately after the infusion of cryopreserved marrow or PBSCs has been described.<sup>55,56</sup> In these two series, the incidence of bradycardia (heart rate <60 beats/minute) was 48.8% and 65%, second-degree heart block was 9.7% and 24%, and complete (third-degree) heart block was 4.8% and 5.9%. In both reports, the median time of onset was about 3 hours after the completion of the infusion. In one series, the authors noted that the heart block was often episodic, occurring with episodes of emesis.<sup>55</sup> In both series, the cardiac rhythm abnormalities resolved spontaneously within 24 hours after infusion. In contrast, another group found no cardiac rhythm changes in a prospective series of 29 patients.<sup>57</sup> A slower overall infusion rate may have accounted for the lack of rhythm changes.

Headache has been reported in up to 70% of recipients of cryopreserved cells,<sup>52</sup> but other central nervous system complications are rare and generally related to the amount of DMSO infused. Two recipients who received HSC components containing a total of 225 mL and 120 mL, respectively, of DMSO developed reversible encephalopathy.<sup>58</sup> The first patient underwent plasmapheresis with prompt improvement in mental status; the second patient recovered over 5 days without specific treatment.

The weights of the patients were not cited in this report, but both patients probably received more than 2 g DMSO per kilogram of body weight. The infusion of large quantities of poorly cryopreserved mature blood cells can cause renal failure.<sup>59</sup> Seizures have been associated with the infusion of highly concentrated PBSC products, suggesting that freezing at very high cell concentrations is not an acceptable strategy to avoid large volumes of DMSO.<sup>49</sup>

### Posttransplant Support

Hematopoietic growth factors are administered to speed neutrophil recovery after allogeneic or autologous transplantation. Randomized studies of granulocyte-macrophage colony-stimulating factor (GM-CSF) or G-CSF administration after autologous HSC transplantation demonstrated that these hematopoietic cytokines speed granulocyte recovery, decrease the morbidity caused by the transplant procedure, and are associated with a smaller probability of relapse.<sup>60-62</sup> But this has not consistently translated into earlier hospital discharge or reduced transplant cost. Administration of cytokines that primarily stimulate the proliferation of mature hematopoietic progenitor cells may not greatly affect the time to initial hematopoietic recovery after transplantation, although the subsequent rate of rise in peripheral blood counts is notably enhanced, suggesting that adequate numbers of cells at the appropriate maturational stage must be present before growth-regulatory effect from these cytokines can be obtained.<sup>63</sup> Late administration of cytokine support (starting several days after HSC infusion) may be as effective as early administration while being less costly to the patient.<sup>64</sup>

### Complications of High-Dose Therapy

The patient undergoing dose-intensive therapy with autologous HSC transplantation experiences a period of marrow hypoplasia that can persist for days or (rarely, with PBSCs) weeks. During this time, the patient requires antibiotic and blood component support, but death from infection or hemorrhage is uncommon.

Common nonhematopoietic toxicity that is not life-threatening includes alopecia, sterility, and varying degrees of mucositis with concomitant mouth pain, inanition, and diarrhea. Rarely, mucositis compromises airway patency, and intubation of the patient is temporarily required for airway protection. Serious organ toxicity includes hepatic veno-occlusive disease (from high-dose regimens in general), interstitial pneumonitis (eg, BCNU-induced), cardiomyopathy (eg, cyclophosphamide-induced), and hemorrhagic cystitis (eg, cyclophosphamide-induced). The risk of nonhematopoietic toxicity increases for older patients and for patients who previously received intensive chemotherapy regimens. For these patients, reduction of dose of the transplant conditioning regimen reduces toxicity but possibly increases the risk of relapse of disease.

Patients may experience long-term marrow hypoplasia after HSC transplantation despite the infusion of large quantities of HSC, possibly as a result of poor cryopreservation techniques, marrow stromal cell damage, or posttransplant events, and this complication may preclude the subsequent administration of

chemotherapy or radiotherapy for patients who experience relapse of disease. The development of myelodysplasia or secondary leukemia is being increasingly reported for patients previously treated with autologous HSC transplantation, particularly for patients with the diagnosis of NHL treated with radiation-containing conditioning regimens.<sup>65</sup> The incidence of this complication appears to be small in patients treated for leukemia,<sup>66</sup> but has been reported above 10% for patients treated for lymphoid malignancies.

Recipients of autologous or allogeneic HSC transplantation are at risk of long-term complications of their treatment including hypothyroidism, hypogonadism, organ toxicity (eg, drug-induced pneumonitis), and secondary malignancies. Patients treated with HSC transplantation should be enrolled in a program of long-term supportive care.<sup>67</sup>

### Adjuvant Therapy with Autologous HSC Transplantation

The incidence of relapse after high-dose therapy leads to the conclusion that high-dose therapy with autologous HSC transplantation, although well tolerated and likely to prolong life, should be viewed as a platform for other approaches that may be effective in eliminating the MRD of patients destined to relapse after the dose-intense therapy is administered. Additional or yet higher-dose chemotherapy or radiotherapy, unless directly targeted to the tumor target, increases the risks of nonhematopoietic toxicity and loss of patients from causes other than relapse. Immunologically based therapies are of interest in this regard and include administration of posttransplant cytokines,<sup>68</sup> the addition of tumor-specific antibodies used before or after transplantation or both as an in-vivo-purge,<sup>69</sup> and the development of tumor-specific vaccines such as with tumor-antigen-pulsed dendritic cells (DCs).<sup>70</sup>

## Diseases

### Acute Myelogenous Leukemia

Induction chemotherapy yields complete remission for most patients with acute myelogenous leukemia (AML), but these patients will rapidly relapse without postinduction consolidation therapy. Randomized studies of consolidation regimens of varying intensity demonstrate that intensity is correlated with probability of durable remissions.<sup>71</sup> The role of autologous or allogeneic HSC transplantation as intensive consolidation therapy for patients entering first remission has been explored in several randomized studies that assigned patients with available sibling donors to allogeneic transplantation and randomly assigned other patients to autologous transplantation or nontransplant consolidation therapy.<sup>72-76</sup> In general, autologous marrow transplantation has not proven to be more effective than nontransplant intensive consolidation chemotherapy (Table 34-2). In a study sponsored by the Eastern Cooperative Oncology Group (ECOG), patients assigned to allogeneic transplantation achieved a 43% DFS compared with 35% for patients assigned to chemotherapy and 35% for patients assigned to autologous transplantation

using marrow cells purged ex vivo with a cyclophosphamide derivative that caused a delay in hematologic recovery.<sup>72</sup> Overall survival (OS) was slightly but significantly higher for chemotherapy-treated patients because of the ability to rescue patients who relapsed, with subsequent autologous transplantation administered in second remission. However, only 54% of patients assigned to autologous transplantation in the ECOG-sponsored study received this treatment, and analysis of outcomes was by intent of treatment, raising the question of whether a difference could have been found with better compliance. Furthermore, transplant-related mortality was 14% for patients assigned to the autologous marrow transplant group, which is higher than would be expected for PBSC transplantation using nonpurged HSC products. A subsequent analysis of treatment outcome for patients stratified by cytogenetic risk group revealed a particularly poor outcome for patients with adverse risk cytogenetics who underwent either autologous HSC transplantation or nontransplant therapy.<sup>77</sup> In contrast, in one study the DFS at 7 years was 54% for recipients of autologous transplantation compared to 40% for recipients of chemotherapy ( $p = 0.04$ ).<sup>73</sup> Another study demonstrated a significantly better DFS for recipients of allogeneic transplantation (48% vs 30%,  $p = 0.04$ ) compared to recipients of chemotherapy. The probability of DFS for recipients of autologous marrow was intermediate, but not significantly different from either of the two other study arms.<sup>76</sup> Therefore, the role for autologous transplantation in the treatment of patients in first remission is not obvious, and questions remain about the selection of HSC source, value of ex-vivo purging, and timing of transplantation relative to other options for postinduction consolidation.

Several Phase II studies of autologous transplantation for the treatment of patients with AML in second or later remission have been published.<sup>78,79</sup> Many of these studies reported survival probabilities of 35% or better, which is higher than what would be expected with standard chemotherapy regimens. A review of British registry data described 154 patients who underwent autologous marrow and/or PBSC transplantation in second remission.<sup>80</sup> At 10 years, the actuarial probability of OS was 32%

**Table 34-2.** Multicenter Randomized Studies Comparing Autologous Marrow Transplantation to Intensive Consolidation Chemotherapy

| Study                      | Probability of Event-Free Survival (%) |            |                           |
|----------------------------|----------------------------------------|------------|---------------------------|
|                            | Allogeneic                             | Autologous | Chemotherapy <sup>†</sup> |
| Zittoun <sup>76</sup>      | 55*                                    | 48         | 30 (4 year)               |
| Cassileth <sup>72</sup>    | 43                                     | 35         | 35 (4 year)               |
| Burnett <sup>73</sup>      | ND <sup>†</sup>                        | 53*        | 40 (7 year)               |
| Harousseau <sup>74</sup>   | 44                                     | 44         | 40 (4 year)               |
| Ravindranath <sup>75</sup> | ND <sup>†</sup>                        | 38         | 36 (3 year)               |

\* Significantly different compared to chemotherapy treatment arm.

<sup>†</sup> Allogeneic transplantation was not performed as part of this trial.

<sup>‡</sup> Shown is the median duration of follow-up at time of analysis.

with PFS. Overall survival was better for patients who experienced a longer duration of first remission and had a younger age at time of transplantation (which may reflect a difference in the biology of this disease for different age groups). Most patients for whom this information was available in this registry had favorable or intermediate risk cytogenetics; only 1% had adverse risk cytogenetics. Autologous HSC transplantation appears to be effective therapy for the patient with AML in second complete remission (CR) who do not have adverse-risk features.

Purging of tumor cells from the HSC inoculum may be beneficial, but remains unproven. In nonrandomized studies, patients who received marrow cells treated with chemotherapy agents were less likely to relapse after transplantation.<sup>81</sup> Similarly, indirect laboratory assessments of purging efficacy suggest a benefit for patients who are treated with an aggressive purging technique.<sup>82,83</sup> The role of purging has not been proven in randomized studies, nor has it been shown to be of benefit for recipients of PBSC.

### Acute Lymphoblastic Leukemia

Few adults with acute lymphoblastic leukemia (ALL) are currently cured with induction and consolidation regimens.<sup>84</sup> Results have improved modestly with more intensive postremission chemotherapy and with tailoring of protocols in individuals with specific subsets of ALL. There are few data, however, supporting the effectiveness of autologous HSC transplantation as currently performed in ALL despite the theoretic potential of dose-intensive therapy. Two published reports comparing autologous to allogeneic marrow transplantation reported DFS after autologous transplantation similar to that reported for patients undergoing less intense induction and maintenance chemotherapy (approximately 20% to 30%).<sup>85,86</sup> A third study comparing autologous to allogeneic transplantation reported significantly improved probability of long-term survival for patients undergoing allogeneic transplantation.<sup>87</sup> Weisdorf et al<sup>88</sup> described transplant outcomes after autologous or unrelated donor transplantation for 712 younger (median age <18 years) patients with ALL (517 unrelated donor, 195 autologous) in first or second CR with data in two registries. They reported much higher treatment-related mortality for recipients of unrelated donor transplants, but more frequent relapses after autologous transplantation in first (49% vs 14%) and second remission (64% vs 25%) leading to similar outcomes for these two transplantation choices. Transplantation in CR1 yielded similar 3-year survival rates for recipients of unrelated donor (51%) and autologous (44%) transplants, as did transplantation in CR2 (40% vs 32%, respectively). As with AML, adverse risk features appears to favor allogeneic transplantation in first remission. A large Phase III study involving 1500 patients with a new diagnosis of ALL comparing specific outcomes for autologous or related-donor allogeneic HSC transplantation or nontransplant consolidation/maintenance therapy has not yet been reported in final analysis, although the study confirmed previously reported risk groups for patients with this diagnosis.<sup>89</sup>

Phase II reports from investigators from the University of Minnesota and Dana-Farber transplant programs reported 56

recipients of 214 adults alive and without disease at a median of 25 months after autologous transplantation.<sup>90</sup> The randomized addition of posttransplant interleukin-2 therapy after autologous transplantation did not improve the DFS. A similar matched-pair study involving children transplanted in second remission reported a 9-year probability of DFS of 26% for patients undergoing autologous transplantation compared to 32% for similar patients treated with chemotherapy alone.<sup>91</sup> In contrast, others reported a 1-year DFS of 50% for adult patients transplanted in first remission and 27% for patients in second or later remission using marrow with or without ex-vivo purging.<sup>92</sup> As with AML, autologous HSC transplantation appears effective for a subpopulation of patients with ALL, particularly those without adverse risk cytogenetic features and (for patients in second remission) who experienced longer durations of first remission.

### Chronic Myelogenous Leukemia

Limited numbers of patients with chronic myelogenous leukemia (CML) in chronic phase or with more advanced disease have been treated with autologous HSC transplantation.<sup>93</sup> In a large, single-center study of 73 patients, the survival of patients with chronic-phase disease who underwent transplantation did not differ from that of patients treated with interferon. Of patients with more advanced disease, 58% achieved a complete hematologic remission but only 10% achieved a complete cytogenetic response, and the median survival for this group of patients was only 5 months. However, other studies have reported longer durations of survival and remission for patients who receive Philadelphia-negative grafts,<sup>94</sup> leading to the hypothesis that products collected from patients in molecular remissions after treatment with imatinib may be useful in dose-intensive therapy for patients with subsequent progression of disease.<sup>95</sup> A meta-analysis of a number of incomplete clinical trials comparing autologous transplantation to nontransplant therapy (principally, interferon-alpha) found no evidence of a difference in survival or statistically significant differences between treatment groups in best hematologic or cytogenetic response achieved in the first year.<sup>96</sup> Furthermore, this analysis was unable to determine whether autologous HSC transplantation with predominantly Philadelphia-negative cells early on in the disease resulted in a better outcome. Autologous HSC transplantation for this disease is, in general, limited to clinical research protocols.

### Chronic Lymphocytic Leukemia

Aggressive treatment is less likely to be used in CLL because of its long natural history, indolent nature, and the advanced age of most patients. Although the disease responds to chemotherapy and radiotherapy initially, relapse is inevitable. The extensive infiltration of marrow by malignant lymphocytes has served to focus transplantation trials on allogeneic transplantation or autologous transplantation with HSC products collected in remission or purged ex vivo. Few clinical studies have been reported. Investigators from the M.D. Anderson Cancer Center reported 6 of 11 patients treated with autologous marrow cells (seven purged

ex vivo) surviving in remission up to 29 months after transplantation.<sup>97</sup> Investigators at the Dana-Farber Cancer Institute reported a high CR response rate for 12 patients who received autologous marrow that had been purged with multiple monoclonal antibodies,<sup>98</sup> but a subsequent report of a total of 137 patients found no plateau in the survival probability, indicating that late relapses continue to occur.<sup>99</sup> Currently, autologous HSC transplantation in the treatment of CLL is limited to clinical research protocols.

Multiple Myeloma

Multiple myeloma, a malignancy of plasma cells, is now the most common indication for autologous stem cell transplantation in North America (Fig 34-1). Autologous stem cell transplantation with dose-intense melphalan is now considered a standard component of the initial therapy of newly diagnosed patients based

on Phase III studies that demonstrated improved response rates and better survival for recipients of this treatment (Table 34-3).

Dose-intense melphalan therapy was first noted to have a dramatic response in nine patients with refractory myeloma in 1983, including five patients who achieved a complete biochemical and marrow remission.<sup>101</sup> Hematopoietic toxicity was initially dose-limiting for dose-intense melphalan, but this could be ameliorated by infusion of previously collected HSCs. The ability to administer high doses of melphalan with or without other drugs or TBI was confirmed in a series of Phase I and II studies. A matched-pair analysis by the University of Arkansas group and Southwest Oncology Group comparing dose-intense with standard-dose therapy demonstrated the benefit of autologous HSC transplantation for the treatment of multiple myeloma with a marked improvement in CR rate (22%) when compared

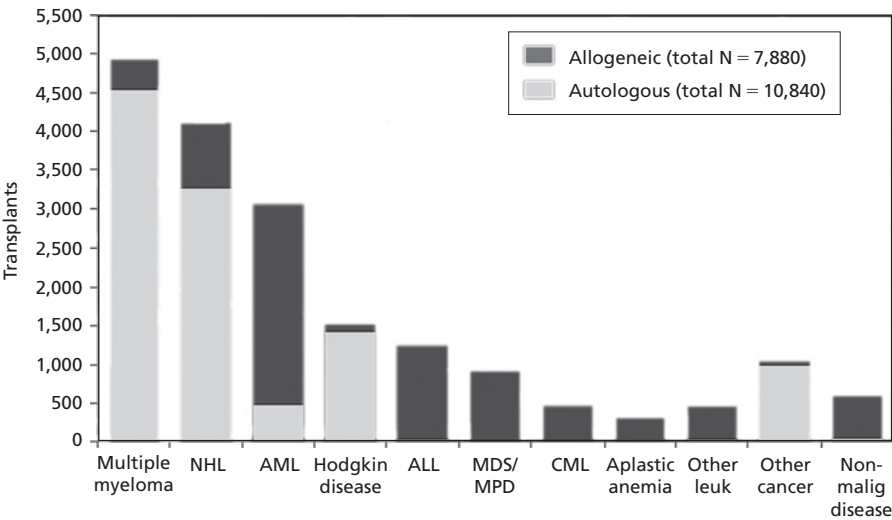


Figure 34-1. Indications for hematopoietic stem cell transplantation in North America in 2005.

Table 34-3. Risk Factors for Survival After Autologous Transplantation for Multiple Myeloma

| Event-Free Survival*       |     |         | Overall Survival           |      |         |
|----------------------------|-----|---------|----------------------------|------|---------|
| Variable                   | RR  | p       | Variable                   | RR   | p       |
| No change in chromosome 13 | 0.5 | <0.0001 | No change in chromosome 13 | 0.4  | <0.0001 |
| B2M ≤2.5 mg/L              | 0.7 | <0.0001 | B2M ≤2.5 mg/L              | 0.6  | <0.0001 |
| ≤12 months therapy         | 0.7 | <0.0001 | ≤12 months therapy         | 0.7  | 0.0001  |
| Sensitive disease          | 0.8 | <0.0001 | CRP ≤4.0 mg/L              | 0.7  | <0.0002 |
| Any 2nd transplant         | 0.8 | 0.0004  | Sensitive disease          | 0.8  | 0.0002  |
| Non-IgA                    | 0.7 | 0.002   | Non-IgA                    | 0.7  | 0.002   |
| Any CR                     | 0.8 | 0.002   | Any 2nd transplant         | 0.04 | <0.0001 |

\* Used with permission from Desikan et al.<sup>100</sup>

B2M = immunoglobulin A; CRP = C-reactive protein; CR = complete remission.



**Table 34-4.** Randomized Trials Comparing Conventional Chemotherapy vs High-Dose Therapy

| Study                       | n   | Age   | Median F/U (months) | CR Rate (%) |     |        | Median EFS (months) |      |         | Median OS (months) |     |        |
|-----------------------------|-----|-------|---------------------|-------------|-----|--------|---------------------|------|---------|--------------------|-----|--------|
|                             |     |       |                     | CC          | HDT | p      | CC                  | HDT  | p       | CC                 | HDT | p      |
| IFM90 <sup>3</sup>          | 200 | <65   | 84                  | 5           | 22  | <0.001 | 18                  | 28   | 0.01    | 44                 | 57  | 0.03   |
| MAG91 <sup>105</sup>        | 190 | 55-65 | 56                  | NE          | NE  |        | 19                  | 25   | 0.05    | 45                 | 42  | NS     |
| PETHEMA <sup>106</sup>      | 164 | ≤65   | 42                  | 11          | 30  | 0.002  | 34                  | 42   | NS      | 67                 | 65  | NS     |
| Italian MMSG <sup>107</sup> | 195 | 50-70 | 24                  | 7           | 26  | <0.001 | 16                  | 28   | 0.0036  | 43                 | 58+ | 0.0008 |
| USIG <sup>103</sup>         | 516 | <70   | 76                  | 15          | 17  | NS     | 21                  | 25   | 0.05    | 53                 | 58  | NS     |
| MRC7 <sup>104</sup>         | 407 | 65    | 42                  | 8           | 44  | <0.001 | 19.6                | 31.6 | <0.0001 | 42                 | 54  | <0.001 |

CC = conventional chemotherapy; CR = complete remission; EFS = event-free survival; F/U = follow-up; HDT = high-dose therapy; OS = overall survival; NE = not evaluated; NS = difference not statistically significant.

to currently available low-dose regimens (5%).<sup>102</sup> Event-free (49 vs 27 months) and overall survivals (62+ vs 48 months) were also improved for patients treated with dose-intense therapy as part of their initial treatments.

Several randomized studies comparing dose-intense therapy with autologous stem cell support to standard therapy regimens have been conducted (Table 34-4).<sup>103</sup> The French Myeloma Intergroup reported a large-scale randomized trial (IFM 90) that randomly assigned 200 patients to either conventional dose VMCP/VBAP polychemotherapy regimen or autologous marrow transplantation with melphalan and TBI after four cycles of induction treatment.<sup>3</sup> The findings were strikingly in favor of the dose-intense approach with improvements in complete response rates (22% vs 5%) and median probabilities of EFS (27 months vs 18 months) and OS (60+ months vs 37 months). The British Medical Research Council (MRC) Myeloma VII trial of 407 patients reported similar results for a comparison of six cycles of BCNU, doxorubicin, cyclophosphamide, and melphalan to at least three cycles of doxorubicin, vincristine, methylprednisolone, and cyclophosphamide followed by melphalan at 200 mg/m<sup>2</sup> and autologous stem cell transplant.<sup>104</sup> The French group Myelome Autogreffe (MAG) achieved results comparable to the IFM 90 trial; however, there was no significant difference in OS as a result of unexpected prolonged survivals in the chemotherapy arm.<sup>105</sup> The Spanish PETHEMA group did not find a statistically significant difference in EFS and OS between chemotherapy and dose-intensive therapy.<sup>106</sup> However, only patients who responded (CR: 15%; partial response: 68%; minor response: 17%) to conventional dose chemotherapy were randomly assigned to treatment groups and patients in the chemotherapy arm were allowed to cross over into the dose-intensive therapy group. The Italian Multiple Myeloma Study Group compared melphalan-prednisone to two courses of melphalan at 100 mg/m<sup>2</sup> with autologous stem cell support.<sup>107</sup> They found significant improvement in CR and EFS in the transplant study arm. The MRC trial comparing high-dose therapy to conventional therapy deserves special consideration.<sup>104</sup> It is the most recent of the randomized trials and was carried out in the era of more advanced

supportive care and the availability of newer therapeutic agents. It still showed a statistically significant advantage for high-dose therapy in terms of CR, EFS, and OS. Moreover, it did not show any increase in treatment-related mortality relative to conventional therapy. These randomized trials all showed a trend to significantly improved results for patients treated with dose-intense therapy. Adding the support of numerous Phase II trials, the data clearly indicate that dose-intense therapy with autologous HSC transplantation should be offered to appropriate patients as part of their initial treatment. The median EFS is remarkably constant (25-31 months). It is more difficult to analyze OS results because that depends partly on salvage therapy. However, median OS was significantly longer in the three studies where differences in EFS were more marked. In all studies, procedure-related death rate was <5% and not greater than that observed with conventional chemotherapy.

With HSC support, mucositis becomes the dose-limiting effect of dose-intense melphalan. In an attempt to increase the total amount of melphalan administered, the Arkansas transplant program explored the feasibility of two consecutive autologous transplants given at intervals of only a few months, doubling the total dose of melphalan administered.<sup>108</sup> The investigators described 42 patients who received two consecutive autologous PBSC transplants using melphalan 200 mg/m<sup>2</sup>. In patients who were capable of receiving two transplants, the CR rate increased from 24% after the first transplant to 43% after receipt of the second transplant. This experience has now been extended in over 1000 patients with overall CR rates of 40% and projected 5-year EFS and OS of 25% and 40%, respectively.<sup>100</sup> The French Intergroup randomly assigned 399 newly diagnosed myeloma patients under age 60 to single vs tandem transplants.<sup>109</sup> They found similar CR rates between the two groups (42% single transplant vs 50% tandem transplant), but superior 7-year EFS (10% vs 20%,  $p = 0.03$ ) and OS (21% vs 42%,  $p = 0.01$ ) for the tandem transplant group. For patients who did not achieve a very good partial response (less than 90% reduction in serum or urine paraprotein) after one transplant, the improvement in 7-year OS was more dramatic (11% vs 43%,  $p < 0.01$ ). These

results support tandem autologous transplants for patients with a diagnosis of multiple myeloma who do not show disease progression after the first transplant.

The success of autologous transplantation led to several analyses of the risk factors predictive for the survival of patients after initial transplantation. A retrospective study of 344 patients with multiple myeloma treated with high-dose chemotherapy and autologous stem cell transplantation showed a 5-year OS of 72% for patients who achieved a complete response, which is defined as absent monoclonal protein by both serum protein electrophoresis (SPEP) and immunofixation (IFE). This is compared to 48% for patients who achieved a near complete remission (monoclonal protein absent on SPEP but still detectable by IFE). The EFS was also superior in the CR group (35%) in comparison to the non-CR group of patients (21%).<sup>110</sup> Investigators from the Arkansas transplant program reported the long-term outcomes for 515 consecutive patients intended to receive melphalan-based tandem transplants with follow-up of at least 5 years.<sup>111</sup> One quarter of the patients had EFS of  $\geq 5$  years with no further relapses seen after 7 years (46 patients on the plateau). Factors associated with long-term EFS included initial transplantation within 1 year of diagnosis, absence of chromosome 11 or 13 abnormalities, and a lower  $\beta_2$ -microglobulin at initial diagnosis. This group subsequently reported that of 135 patients enrolled in a study of tandem autologous HSC transplantation, patients with normal cytogenetics had median EPS and OS of 43 and 50 months or more, respectively.<sup>112</sup> The EFS and OS for patients with abnormalities of chromosome 13 were 21 and 29 months; for patients with abnormalities involving 11q, 20 and 21 months; and for patients with abnormalities of both 13 and 11q, 11 and 12 months. The absence of chromosomal abnormalities was the single strongest prognostic factor associated with prolonged survival in this study. Other factors that are considered poor prognosticators are fluorescence in situ hybridization (FISH)-defined chromosome 13 deletions<sup>113</sup>; hyperdiploidy<sup>114</sup>; the detection of abnormal metaphases<sup>100</sup>; elevations in C-reactive protein, lactate dehydrogenase,  $\beta_2$ -microglobulin, and creatinine; and low levels of hemoglobin, albumin, and platelets.<sup>115</sup>

The role of maintenance therapy after transplantation remains open to question. A recently published study assigned 597 patients to three arms of (A) no maintenance therapy, (B) pamidronate therapy given monthly, or (C) pamidronate with thalidomide starting 2 months after a second autologous HSC transplantation.<sup>116</sup> Those who received pamidronate and thalidomide (Arm C) had the best CR or very good partial response when compared to the Arm A and Arm B patients (67% vs 55% and 57%, respectively). The 3-year probability of relapse-free survival was also higher in Arm C vs Arm A and B (51% vs 38% and 39%, respectively); 87% compared to 77% and 74%, respectively. Of note, the thalidomide therapy showed no benefit in patients with chromosome 13 deletion or those who achieved least a very good partial response to the dose-intense therapy. Additional studies of this question are in progress.

## Amyloidosis

Primary amyloidosis is a plasma cell dyscrasia in which clonal plasma cells in the marrow produce a monoclonal immunoglobulin protein. These M-protein light chains or light-chain fragments form insoluble fibrils that are deposited into the extracellular matrix of a variety of tissues, resulting in severe multi-organ dysfunction and poor patient survival. Randomized studies have shown that melphalan-based therapy can prolong the life of patients with primary amyloidosis.<sup>117</sup> Dose-intense therapy with melphalan and autologous PBSC transplantation can induce a complete hematologic remission, defined as the absence of detectable plasma-free light-chain protein.<sup>118</sup> Responders who achieve a complete disappearance of light-chain secretion are more likely to achieve reversal of underlying organ dysfunction.<sup>119</sup>

Patients frequently present with extensive systemic disease, rendering this disease difficult to treat. Regimen-related mortality is close to 20% of patients in some studies.<sup>120</sup> Multi-organ disease (particularly cardiac dysfunction) predicts a poor transplant outcome. In one study, the EFS was only 60% for patients with cardiac involvement but 100% for patients with renal involvement and no evidence of cardiac dysfunction.<sup>118</sup> Single center studies, particularly with adjustment of melphalan dose for patients with higher risk of toxicity, report a much lower mortality incidence. This suggests that the use of dose-intense therapy should be limited to centers with greater experience in the treatment of this disease.<sup>121</sup> A randomized study conducted in Europe did not find improved survival for patients treated with PBSC transplantation, but the transplant arm was characterized by a high mortality rate that could obscure the benefit of dose-intense therapy for selected patients.<sup>122</sup> As with the treatment of multiple myeloma, one group found that tandem cycles of dose-intense melphalan with autologous PBSC support improved the response rate for patients with systemic amyloidosis.<sup>123</sup> Thalidomide and lenolidamide are also effective therapies in the treatment of amyloidosis with a small proportion of patients achieving a complete hematologic response. These agents may be useful in the pre- or posttransplant management of patients with amyloidosis.<sup>124,125</sup>

## Lymphoma

The lymphomas represent a diverse group of hematologic malignancies arising from lymphoid tissues. They vary from the slowest to some of the fastest growing tumors. They also range from highly curable to essentially incurable. The lymphomas are highly responsive to chemotherapy and radiation in most cases. These tumors exhibit a strong dose-response relationship, and the benefit of high-dose treatment with autologous stem cell rescue is well established for some categories of this disease.

Several high-dose chemotherapy regimens have been developed for the treatment of the lymphomas. Radiation-based regimens including cyclophosphamide with TBI and etoposide plus TBI have significant activity but frequently are difficult to administer in patients already exposed to radiotherapy. Popular

chemotherapy regimens include cyclophosphamide, BCNU, and etoposide (CBV) and BCNU, etoposide, cytarabine, and melphalan (BEAM). However, no single chemotherapeutic regimen has emerged as a superior treatment.

### Hodgkin Disease

Many patients with Hodgkin disease will achieve durable remissions with nontransplant chemotherapy with or without radiation therapy, and algorithms for staging and treatment of this disease are well defined. Dose-intense therapy with autologous transplantation should be considered for those patients who do not achieve a remission or who relapse after initial therapy and will succumb without aggressive therapy.<sup>4,126</sup> The inclusion of dose-intense therapy as part of the initial therapy of patients with adverse-risk features is not supported in clinical studies.<sup>127</sup>

For patients who suffer a relapse after achieving CR, the prognosis with conventional salvage therapy is directly related to the duration of the initial remission. The outcome for patients whose remissions lasted less than 1 year is dismal with standard-dose second-line treatments, and most experts concur that these patients are best treated with high-dose chemotherapy with stem cell rescue. Approximately 40% to 50% of patients with Hodgkin disease who suffer a relapse within 1 year will achieve durable remissions after autologous HSC transplantation.<sup>128-130</sup> For patients whose first remission lasts more than 1 year, both conventional-dose chemotherapy and high-dose autologous stem cell therapy provide the possibility of overall survival, although HSC transplantation favors PFS.<sup>131</sup> Two randomized trials comparing high-dose chemotherapy with conventional salvage treatment have been performed.<sup>132,133</sup> These studies suggest similar overall long-term survival rates but possibly a better DFS rate for the transplant arms. The British national lymphoma trial compared high-dose BEAM-with-transplant with mini-BEAM salvage therapy. Accrual was terminated prematurely because patients refused randomization and requested the high-dose therapy. Patients who underwent autologous transplantation had statistically greater EFS and PFS rates. Overall survival rates, however, were the same in both groups in this very small trial. A second European Group for Blood and Marrow Transplantation (EBMT) trial comparing standard-dose with high-dose treatment revealed improved time to treatment failure with the autologous arm but, again, overall survival in this slightly larger trial was not yet improved. Numerous risk factors are now identified that predict lower rates if disease control after HSC transplantation is achieved in the treatment of Hodgkin disease. These risk factors include chemotherapy-resistant disease, more than minimal residual disease at the time of transplant, B symptoms at relapse, poor performance status, relapse within a prior radiation field, and high International Prognostic Factor Project score.<sup>134,135</sup>

Patients with refractory Hodgkin disease may achieve durable complete remissions with high-dose chemotherapy and stem cell rescue. Numerous series, including that from the Autologous Blood and Marrow Transplant Registry [ABMTR—now the Center for International Blood and Marrow Transplant Research

(CIBMTR)] demonstrate that high-dose treatment can overcome drug resistance in Hodgkin disease. In the ABMTR analysis, patients were considered to have primary refractory Hodgkin disease if they never achieved CR. Following transplantation, the probability of 3-year PFS was 38% with an OS rate of 50%.<sup>136</sup> Results from an EBMT Registry analysis were similar to that in the American series. The European group reported an actuarial 5-year DFS rate of 32% and an OS rate of 36% for patients with refractory Hodgkin disease.<sup>126</sup>

### Low-Grade Non-Hodgkin Lymphoma

Low-grade NHLs, in general, exhibit variable and prolonged natural courses, with many patients not requiring treatment until symptoms or organ toxicity appear. Therefore, most of the experience with HSC transplantation has been in patients after initial relapse rather than at the time of initial diagnosis, avoiding the potential for morbidity and mortality of dose-intense therapy. Multiple studies have demonstrated a greater response after dose-intense chemotherapy with autologous HSC transplantation compared to combination chemotherapy rescue regimens in the treatment of advanced or relapsed follicular lymphoma. Patients enrolled in a European Phase III randomized trial received dose-intense therapy and marrow transplantation (with or without ex-vivo purging). They benefited from a longer PFS compared to patients who were randomly assigned to the more standard-dose chemotherapy arm (55-58% vs 26%) and a significantly longer OS at 4 years (71-77% vs 46%).<sup>137</sup> The benefit of dose-intense therapy for newly diagnosed patients is less obvious. A German study enrolled 240 newly diagnosed patients with advanced follicular lymphoma in a Phase III randomized trial comparing myeloablative therapy followed by autologous HSC transplantation to combination chemotherapy with interferon.<sup>138</sup> At a median follow-up of 4.2 years, the myeloablative therapy arm was found to have a much longer 5-year PFS (65% vs 33%). However, this treatment arm also had higher acute toxicity associated with it and a higher 5-year risk of developing other hematologic malignancies such as treatment-related myelodysplasia (3.8% vs 0%). In contrast, 401 newly diagnosed patients with advanced follicular lymphoma were also studied in the GELA randomized study that compared dose-intense chemotherapy and TBI with autologous HSC transplantation to combination chemotherapy with interferon and found no significant difference in EFS or OS between the two arms after a median follow-up of 7.5 years.<sup>139</sup>

Although response rates are high, a continuing pattern of relapse has been observed with autologous transplant. One group reported a 68% CR rate for a single-center series of 100 patients with relapsed disease but, at a median of 4 years, a probability of failure-free survival of 44% with no plateau to the curve depicting probability of relapse.<sup>140</sup> The Dana-Farber transplant program reported a series of 86 patients with advanced follicular lymphoma who underwent autologous marrow transplantation as part of initial therapy. Their publication raised the question about the necessity for and efficacy of tumor cell purging.<sup>141</sup>

Patients who received marrow products that were free of disease (based on a sensitive PCR detection technique for the t14;18 abnormality commonly found in follicular non-Hodgkin lymphoma) experienced a much lower rate of relapse than similar patients whose products contained detectable tumor after purging. However, no plateau to the curve depicting probability of relapse was reported. Patients who undergo allogeneic transplantation will experience a higher probability of transplant-related mortality but a lower risk of relapse after transplantation.<sup>142</sup> The difference in relapse rates could result either from reintroduction of lymphoma cells in the HSC product or, more likely, from the lack of the powerful and beneficial graft-vs-disease effect of allogeneic HSC transplantation.

Small numbers of patients have undergone autologous HSC transplantation for aggressive non-Hodgkin lymphoma after transformation from indolent non-Hodgkin lymphoma,<sup>143,144</sup> with several reports describing improved survival for patients treated with dose-intense therapy compared to standard combination chemotherapy regimens. In patients under the age of 60 with chemotherapy-sensitive disease, the 4- to 5-year DFS and OS were reasonable (30%-52% and 37%-63%, respectively),<sup>144</sup> suggesting that this therapy should be considered for this group of patients.

### Aggressive Non-Hodgkin Lymphoma

The standard of care for patients with B-cell non-Hodgkin lymphoma in first "chemotherapy-sensitive" relapse is HSC transplantation.<sup>5,145-147</sup> The success of this therapy reflects the extent and the responsiveness of the disease to chemotherapy at the time of transplantation.<sup>148,149</sup> Relapse is the major cause of failure of autologous transplantation.

The role of high-dose chemotherapy with stem cell rescue in patients with chemotherapy-sensitive aggressive (intermediate or high-grade) lymphomas in relapse has become well established. The Parma trial randomly assigned 109 patients in first chemotherapy-sensitive relapse after two cycles of salvage chemotherapy to either high-dose therapy or four additional cycles of conventional-dose treatment.<sup>5</sup> The EFS at 5 years was superior for the group undergoing high-dose therapy compared with the group receiving standard-dose treatment (46% vs 12%,  $p = 0.001$ ). The same superiority for transplant was true of 5-year OS (53% vs 32%,  $p = 0.038$ ). Furthermore, it is notable that in the Parma trial no patients assigned to the conventional-dose salvage therapy could be rescued at the time of second relapse with a delayed transplant. Therefore, excessive pretransplant "debulking" therapy with or without delays in transplant timing should be avoided. This randomized trial confirmed previous Phase II studies and conclusively demonstrated that transplantation therapy represents the treatment of choice for most patients with chemotherapy-sensitive first-relapsed aggressive non-Hodgkin lymphoma.

Hematopoietic stem cell transplant may also be indicated for patients who respond slowly to initial therapy, have high-risk disease, are resistant to initial therapy, and are in relapse with chemotherapy-insensitive disease. The appropriate approach

for patients who achieve less than a complete response or who exhibit a slow response to conventional induction therapy is not yet clear. In one small trial of 69 slow responders to CHOP chemotherapy (cyclophosphamide, doxorubicin, and vincristine along with prednisone), the use of early transplant compared to five additional cycles of CHOP was not associated with an overall improvement in survival or EFS ( $p < 0.10$ ).<sup>150</sup>

The International Prognostic Index (IPI) identifies patients with aggressive non-Hodgkin lymphoma who have a high likelihood of relapse and poor overall survival with conventional first-line therapy.<sup>151</sup> The French LNH-87 trial examined the role of consolidation transplantation for patients in CR1 after standard-dose treatment.<sup>146,152</sup> The initial analysis of this trial demonstrated no advantage gained by the addition of intensive consolidation. However, subsequent analysis that included only those patients with intermediate or high risk by the more restrictive criteria of the IPI, revealed a superior DFS for those patients undergoing high-dose transplantation. Similarly, the Italian Non-Hodgkin Lymphoma Study Group showed no benefit from high-dose therapy with stem cell rescue in patients judged to be at high risk by virtue of tumor bulk or advanced-stage disease.<sup>153</sup> However, a striking advantage in DFS was noted when the 70 patients who qualified as high-intermediate or high risk based on the IPI were analyzed. In this subgroup, transplantation yielded a superior 6-year DFS rate (87% vs 48%,  $p = 0.008$ ). Autologous transplantation therapy is likely to be beneficial in patients with high-risk disease as measured by IPI.

Analysis of ABMTR data showed that among 184 patients who had never achieved a complete remission with conventional therapy (primarily induction failures), 44% could achieve one after autologous marrow or PBSC transplantation.<sup>154</sup> The probability of PFS 5 years after transplantation was 31%. Chemotherapy resistance, administration of multiple cycles of chemotherapy before transplantation, poor performance status at time of transplantation, older age, and the lack of use of consolidative radiotherapy before or after transplantation predicted poor outcome.

Patients with disease that is unresponsive to chemotherapy at the time of relapse have a low (about 15% to 20%) chance of achieving a long-term remission with high-dose therapy and stem cell rescue.

Mantle cell lymphoma is known for its unremitting clinical course when treated conventionally and has proven relatively resistant to high-dose treatment with autologous HSC support, especially when used as a salvage therapy.<sup>155</sup> Incorporating high-dose therapy into the initial treatment plan, however, may be more likely to provide durable remissions. At M.D. Anderson Cancer Center the results of using an intensive induction regimen followed by autologous transplantation have been encouraging.<sup>156</sup> Among previously untreated patients, the EFS rate at 3 years was 72%. Mantle cell NHL is very responsive to the graft-vs-disease effect of allogeneic transplantation, and this treatment approach should be given priority over autologous HSC transplantation.

Burkitt lymphoma, Burkitt-like lymphomas, and lymphoblastic lymphomas are eminently curable in most children. However, these high-grade non-Hodgkin lymphomas are associated with relatively poor long-term survival rates in adults. Data from the EBMT Registry suggests that disease status at the time of transplant is the most important predictor of outcome in patients with high-grade disease.<sup>149</sup> For Burkitt and Burkitt-like lymphoma, the 3-year actuarial OS rate was 72% for patients who received a transplant during CR1, compared with 37% in those with chemotherapy-sensitive relapse, and 7% in patients with disease that is unresponsive to chemotherapy. For patients with lymphoblastic lymphoma, the 6-year actuarial survival rate ranged from 63% in patients who were in first CR1 to 15% in those who had resistant disease. Patients in CR2 had an intermediate survival rate of 31% at 6 years. These results with transplantation are superior to conventional-dose salvage therapy.

### Waldenström Macroglobulinemia

Waldenström macroglobulinemia is a rare lymphoproliferative disorder characterized by the production of IgM paraprotein. Waldenström macroglobulinemia, despite the paraprotein production, is distinct in its natural history and response compared to multiple myeloma, with alkylating agents, nucleoside analogues, and rituximab all being effective in the initial treatment of this disease. Only limited numbers of patients who have undergone autologous (or allogeneic) transplantation are described in single center or registry reports but, despite this limitation, a recent workshop on the treatment of patients with this disease recommended collection of autologous HSC before extensive therapy that would limit the ability to collect adequate products.<sup>157</sup> In a Phase I/II study of autologous transplantation, seven patients (untreated or after first-line therapy) with symptomatic disease underwent two or three cycles of cytoreductive chemotherapy with collection and purging of PBSC followed by autologous transplantation after conditioning with TBI and high-dose cyclophosphamide.<sup>158</sup> Engraftment was prompt, without any procedure-related deaths. A strong reduction or normalization of the marrow infiltration and serum IgM levels occurred in all evaluable patients, but immunofixation electrophoresis revealed persistent paraproteinemia in five. A registry report of 10 patients who underwent autologous transplantation reported a PFS at 3 years of 65% with an OS of 70%.<sup>159</sup>

### Solid Tumors

Autologous transplantation for the treatment of solid tumors is less established, primarily because most malignancies do not exhibit the strong dose-response to myelosuppressive chemotherapeutic agents. Exceptions are germ cell tumors and some pediatric malignancies such as neuroblastoma.

### Breast Cancer

Breast cancer was at one point the most frequent indication for autologous HSC transplantation.<sup>160</sup> However, this treatment approach is no longer considered appropriate. The experience of

Bonadonna and others showing a dose response for this malignancy led investigators in the early 1990s to explore the use of dose-intense chemotherapy regimens for women with micrometastases involving 10 or more axillary lymph nodes and for women with inflammatory breast cancer. Peters et al<sup>161</sup> treated 102 women with high-risk ( $\geq 10$  axillary lymph nodes positive for disease) Stage IIA, IIB, IIIA, or IIIB cancer with four cycles of standard-dose CAF (cyclophosphamide, doxorubicin, fluorouracil) chemotherapy followed by high-dose STAMP-1 (cyclophosphamide, BCNU, and cisplatin) chemotherapy with autologous stem cell rescue. The actuarial EFS for the study population at 2.5 years was 72%. Comparison to three historic or concurrent Cancer and Leukemia Group B adjuvant chemotherapy trials selected for similar patients showed an EFS at 2.5 years to be between 38% and 52%. However, several randomized studies did not demonstrate a survival advantage for patients treated with dose-intense therapy compared to those treated with more standard-dose treatments.<sup>162-165</sup> Dose-intense therapy with HSC transplantation also does not appear to provide a survival advantage for women with metastatic disease.<sup>166</sup>

### Germ Cell Tumors

Germ cell cancers are relatively uncommon diseases accounting for about 1% of all malignancies in primarily adolescent and young men. Germ cell tumors, highly curable with nontransplant therapies, are classified as seminomatous or nonseminomatous tumors. Despite a propensity for metastatic spread, germ cell cancers are one of the most highly curable human malignancies. Over 60% of patients with high-risk disseminated disease will achieve durable responses after treatment with four cycles of cisplatin, etoposide, and bleomycin. Patients with refractory disease or who relapse after initial treatment can be effectively rescued with one or two cycles of dose-intense therapy.

Forty cisplatin-refractory patients were entered into a large multi-center Phase II (ECOG-sponsored) trial of carboplatin and etoposide, of whom 22 (58%) were able to proceed to a second cycle.<sup>167</sup> Five patients (13%) died of treatment-related causes including infection, hemorrhage, and hepatic veno-occlusive disease. All treatment-related deaths occurred during the first course of therapy. Nine patients (24%) achieved a complete response (including two patients after surgical resection of residual disease). Another eight patients achieved a partial response for an overall response rate of 45%. Three of the complete responses occurred after the first marrow transplant, and for four patients the partial response converted to complete response after the second marrow transplant. Five of the nine patients were alive and free of disease at a minimum follow-up of 18 months. A striking finding of this study was the poor outcome in patients with nonseminomatous primary mediastinal germ cell tumors. Eleven patients with this diagnosis were enrolled in this study and none obtained a durable complete remission.

The initial ECOG experience with multiple cycles of high-dose therapy has been replicated at single centers. The Memorial Sloan Kettering group treated 58 patients with refractory germ

cell tumors with two cycles of high-dose carboplatin, etoposide, and cisplatin. Forty percent achieved a complete response, with a 2-year survival rate of 31%. Patients with pretreatment beta human chorionic gonadotropin ( $\beta$ -hCG) values below 100 and those without retroperitoneal masses fared better.<sup>168</sup> In contrast, this same group reported that the inclusion of dose-intense therapy did not improve survival for newly diagnosed patients with poor-risk features, although there was a trend for better survival for patients not showing a rapid response to initial treatment who then received dose-intensification with HSC support.<sup>169</sup>

The Indiana University group reported 184 patients with seminomatous and nonseminomatous germ cell tumors who had progressed following cisplatin-based regimens.<sup>170</sup> Patients received one or two cycles of conventional-dose salvage, followed by one ( $n=11$ ) or two ( $n=173$ ) cycles of high-dose carboplatin and etoposide with stem cell support. At a median of 48 months of follow-up, 116 patients remain progression free, including patients with cancer refractory to standard doses of cisplatin. This very aggressive schedule resulted in one of the highest response rates noted to date.

### Ovarian Carcinoma

Autologous transplantation for the treatment of epithelial (non-germ-cell) ovarian carcinoma remains under active investigation, and promising results have been reported for some groups of women. A review of registry data for 421 patients undergoing transplantation identified a better outcome for patients with platinum-sensitive, low-bulk disease with the authors recommending prospective clinical studies.<sup>171</sup> However, one reported Phase III study that enrolled 149 previously untreated women with epithelial ovarian cancer found no differences in EFS or OS, indicating that further studies are needed.<sup>172</sup>

### Other Diseases

The improved safety of autologous PBSC transplantation encourages studies of dose-intense therapy in the treatment of nonmalignant diseases, including autoimmune disorders such as multiple sclerosis, scleroderma, systemic lupus erythematosus (SLE), Crohn disease, and refractory rheumatoid arthritis.<sup>173</sup> The hypothesis of currently ongoing studies is that dose-intense lymphoablative conditioning regimens will achieve prolonged remission or cure of these diseases by destruction of the clone of cells responsible for the disease. This hypothesis is supported by reports of remission of autoimmune diseases (eg, rheumatoid arthritis, SLE) that existed as a comorbid condition for patients undergoing autologous or allogeneic transplantation,<sup>174,175</sup> although this has not been a universal finding.<sup>176</sup> The use of intensive chemoradiotherapy with HSC rescue offers an opportunity to deliver maximally tolerated immunosuppression. Factors that may influence the relative efficacy of this approach include the number and relative resistance to ablation of the responsible lymphoid effector populations and the nature and persistence of the relevant antigen against which the immune response is raised. Although lymphoid effectors are not well defined for

these diseases, evidence points to the involvement of T and B lymphocytes. High-dose therapy followed by autologous HSC transplantation may allow the immune system to “reset” with control of the autoreactive lymphocytes. As with the treatment of malignant diseases, depletion of the unwanted lymphoid cell population from the HSC inoculum (purging) might be necessary for optimal clinical outcome. These nonmalignant diseases are debilitating, and appropriate patient selection is necessary so that the risks (morbidity and mortality) are appropriately balanced against the benefits (possibly, lack of progression instead of reversal of damage already incurred) of this therapy.

Multiple centers have now published their experiences in the treatment of small numbers of patients indicating that this treatment may be effective in controlling autoimmune diseases in certain patient populations. Burt et al<sup>177</sup> recently reported 50 patients who received a nonmyeloablative conditioning regimen of high-dose cyclophosphamide and equine antithymocyte globulin with autologous PBSC support in the treatment of SLE. The treatment-related mortality was 4% (2/50; one death occurring after mobilization of PBSC and one after transplantation), but the probability of 5-year OS was 84%, and the probability of DFS was 50%. These patients also demonstrated stabilization of renal and pulmonary functions and significant improvement or stabilization in markers of disease activity. Similarly, 73 patients were identified from registry data as having received autologous HSC transplantation for the treatment of rheumatoid arthritis, with 49 patients (67%) achieving at least a 50% response at some point after transplantation. There was a significant reduction in the level of disability. Most patients restarted other therapies within 6 months for persistent or recurrent disease activity. This provided disease control in about half the cases, suggesting that the transplant may have ameliorated the disease.<sup>178</sup> These publications set the stage for larger Phase II and III clinical studies to determine proper patient selection and choice of treatment strategies.

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## Summary

Autologous HSC transplantation with dose-intense therapy is now a standard treatment for many diseases. Improved safety of this therapy has increased acceptance by patients and allowed its extension to older patients and those with comorbid diseases. The major failing is the difficulty in curing patients with advanced or refractory diseases. Older patients face a higher risk of relapse that appears to be a result of differing sensitivity of their diseases to dose-intense therapy. This suggests the need for a different approach based on age. Posttransplant immunologic approaches now being studied may, however, be able to convert the minimal disease remaining after transplantation into a cure. Continued effort will determine if modifications of pretransplant conditioning regimens, choice of HSC source, ex-vivo processing, or supplementation of the cell inoculum with other myeloid or lymphoid cells affects engraftment kinetics or immunologic

reconstitution, and thereby enhances the therapeutic value of autologous transplantation for patients with malignant and non-malignant diseases.

## Disclaimer

H. Rashidi has disclosed no conflicts of interest. S. Rowley has disclosed a financial relationship with Johnson and Johnson.

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# Hematopoietic Progenitor Cells: Allogeneic Transplantation

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In 1957 Thomas and colleagues published the first reported attempts at marrow transplantation in six patients with advanced malignancies who were given marrow after receiving limited doses of chemo- or radiotherapy.<sup>1</sup> For the next decade this therapy was limited to patients with terminal leukemia or severe marrow failure resulting from radiation exposure or disease. Although these first efforts provided groundwork for later animal and human studies, success was minimal with almost all of these early patients dying from complications of graft failure, graft-vs-host disease (GVHD), infections, or their primary disease. It was not until 1968 that the first successful allogeneic marrow transplant was reported, in a patient with severe combined immunodeficiency.<sup>2</sup> As a result of the pioneering work of Thomas and colleagues, the number of allogeneic transplants began to increase dramatically over the next decades into the established specialty it is today.<sup>3,4</sup> This chapter provides a brief overview of hematopoietic stem cell transplantation (HSCT) including the types of transplants, key procedural elements, donor sources, disease indications, and potential complications.

## Hematopoietic Stem Cell Transplantation

HSCT can be divided into three main types: 1) autologous transplantation, in which a patient serves as a self-donor, 2) syngeneic transplantation wherein the donor is a genetically identical twin, and 3) allogeneic transplantation wherein the donor is genetically different from the recipient. Marrow transplantation was initially conceived as a means for allowing hematopoietic recovery following the administration of dose-intensive chemo- and radiotherapy for treatment of malignancies. For autologous and syngeneic transplants, the therapeutic role remains hematopoietic cellular rescue following high-dose therapies. In comparison, for allogeneic

transplants, the infused cells result in an additional therapeutic potential arising from a powerful donor immune-mediated response against the host malignancy, a phenomenon referred to as graft-vs-tumor (GVT) effect. Numerous lines of evidence support the therapeutic potential in the GVT effect and are detailed in Table 35-1.<sup>5-10</sup> Although a GVT benefit does not occur with autologous transplants, these types of grafts are associated with less risk because the infused cells will not be immunologically rejected or mediate an immunologic reaction. In comparison, allogeneic transplants have a greater rate of complications because of the potential for graft rejection, or more commonly, GVHD.

### Procedure Fundamentals: Autologous Transplantation

Autologous transplantation is a strategy in which high-dose therapy is administered to patients with a malignancy known to be dose-responsive. In order to receive an autologous HSCT, the patient must first undergo stem cell collection, followed by cryopreservation and storage of the marrow or peripheral blood progenitor cells (PBPCs). Once marrow or PBPCs are successfully collected, the patient can receive intensive myeloablative chemo- or radiotherapy (or both), followed by infusion of the previously

**Table 35-1.** Evidence Substantiating a Graft-vs-Tumor Effect

1. Relapse is lower in allogeneic transplants when compared to autologous or syngeneic transplants.
2. T-lymphocyte-depleted transplants have a higher incidence of relapse when compared to T-lymphocyte-replete transplants.
3. Relapse rates are inversely associated with acute graft-vs-host disease (GVHD) incidence and severity.
4. Relapse rates inversely associated with incidence of chronic GVHD.
5. Delayed clearance of minimal residual disease following allogeneic transplantation (beyond the timeframe in which it could be attributed to cytotoxic therapy administered with the conditioning regimen).
6. Induction of remission following withdrawal of immunosuppression after transplantation in a minority of cases.
7. Induction of remission following infusion of donor lymphocytes.

cryopreserved cells to restore hematopoiesis. Autologous HSCT is discussed thoroughly in Chapter 34. Its brief description here is for ease of comparison with allogeneic transplantation.

### Progenitor Cell Collection

Ideally, the collection process should occur at a time when the marrow has normal cellularity and a limited number of malignant cells. The resting marrow contains both hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs). HSCs are defined as clonal precursor cells that are capable of self-renewal as well as giving rise to a defined set of differentiated progeny. HPCs lack the ability for self-renewal but, similar to HSCs, have the ability to differentiate and proliferate.<sup>11,12</sup> The ability of the stored product to engraft depends on the presence of both types of cells. This chapter collectively refers to these cells as HPCs, which can be identified by the cell expression of CD34 and Thy-1 and the absence of Lin and CD38 cell markers.<sup>11,13</sup>

Historically, HPCs capable of reconstituting hematopoiesis were collected through harvesting the marrow via multiple aspirations from sites such as the ilium. More commonly today, HPCs are collected from the peripheral blood by leukapheresis. In this procedure, the normally low levels of circulating HPCs are increased through the use of hematopoietic growth factors such as granulocyte colony-stimulating factor (G-CSF) alone or in combination with mobilizing doses of cytoreductive chemotherapy (termed chemomobilization).<sup>14,15</sup> Chemomobilization strategies generally result in a higher yield of HPCs when compared to strategies using hematopoietic growth factors alone.<sup>16</sup> PBPC collections have now largely replaced marrow harvesting procedures because of the ease and safety (sparing the need for general anesthesia) of the procedure. In addition, PBPC transplantation results in quicker recovery of granulocytes and platelets than does marrow transplantation.<sup>17</sup> Quantification of CD34-expressing cells in the PBPCs is often performed to assess the probability of successful reconstitution of hematopoiesis upon infusion.<sup>18</sup> Threshold levels resulting in timely neutrophil and platelet recovery range from 3 to  $5 \times 10^6$  CD34+ cells/kg body weight.<sup>19</sup>

### Preparative Regimens

The combination of chemo-, radio-, and biologic therapies in preparation for HSCT is referred to as a preparative (or conditioning) regimen. The sole role of the preparative regimen in the autologous transplant setting is eradication of any residual cancer. High-dose therapy is employed to overcome tumor resistance. In addition, the combination of chemotherapies may provide a way to act on different targets within cancer cells.<sup>20</sup> Creation of regimens is generally based on the principles of minimizing overlapping toxicity and mechanisms of resistance. Dose escalation achievable with the combination regimens is usually less than with each drug alone. Candidate drugs for incorporation into preparative regimens are those agents with a steep dose-response curve and myelosuppression as their dose-limiting toxicity. However, dose escalation is not feasible when

extramedullary toxicity occurs at lower doses or at the same dose levels as myelosuppression. For this reason, alkylating agents are commonly employed. For instance, the dose of thiopeta can be escalated significantly with stem cell support.<sup>21,22</sup> Conversely, adriamycin is seldom used because the mucositis and cardiac toxicity associated with dose escalation of this drug cannot be overcome by the use of HPCs.

Numerous conditioning regimens have been evaluated for various diseases with choice of regimens often based on the preference and experience of the clinician. High-dose chemoradiation therapy for multiple myeloma and lymphoma, two of the most common indications for autologous transplantation, is reviewed later in this chapter. Recently, the addition of newer “targeted” therapies to conditioning regimens has been explored as a way of increasing efficacy and minimizing toxicity. Monoclonal antibodies and radioconjugates for B-cell lymphomas and certain leukemias are examples of newer therapies that have been added safely to conditioning regimens and may offer additional therapeutic benefit with little or no increase in toxicity.<sup>23,24</sup>

Autologous transplantation is a relatively safe procedure with minimal treatment-related mortality. There is no specific age cut-off for the procedure, and patients well into their seventh and eighth decades of life safely undergo the therapy.<sup>25,26</sup> Specific disease indications and complications are discussed later in the chapter.

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## Allogeneic Transplantation

The most obvious difference between autologous and allogeneic HSCT is the need for a donor in the latter. Historically, most allografts used marrow cells obtained from an HLA-matched sibling. However, many potential candidates for this therapy lack a matched sibling donor and transplant procedures using cells obtained from volunteer unrelated donors, mismatched family members, and cord blood are becoming more common. HLA compatibility is the strongest predictor for the occurrence of severe GVHD and is the single most important factor to consider in selecting an allogeneic donor.<sup>27</sup>

The major histocompatibility complex (MHC) is located at p21.3 on the short arm of chromosome 6, where several closely linked genes referred to as the HLA system are located.<sup>28</sup> These genes have been subdivided into Class I loci, which includes HLA-A, -B, and -C, and Class II loci, which includes HLA-DR, -DQ, and -DP. For HSCT, HLA-A, HLA-B, and HLA-DR are routinely evaluated.<sup>29,30</sup> Matching at HLA-C has been confirmed to have an impact on outcome.<sup>27</sup> In its strictest sense, HLA identity means that the donor and recipient are matched for the amino acid sequence encoded by all HLA loci. It is important to note that conventional typing techniques detect a limited number of HLA polymorphic sequences. Therefore, “HLA matched” may not be “HLA identical.” HLA genotypically identical siblings inherit the same MHC haplotypes and, therefore,

are also identical for other non-HLA genes and polymorphisms that are carried on those haplotypes. This is unlike the case for unrelated donors and patients who, although matched for Class I and II alleles, may not necessarily be identical for non-HLA polymorphisms. However, to date studies have not been able to substantiate an effect on outcome for mismatching at any of the known microsatellite polymorphic regions tested.<sup>31</sup>

Conventional serologic typing is based on the complement-dependent microlymphocytotoxicity test and uses selected HLA-specific alloantisera or monoclonal antibodies to identify HLA antigens.<sup>32</sup> Mismatching between cross-reactive antigens is considered a minor mismatch, whereas mismatching between non-cross-reactive antigens is considered a major mismatch. For related patient-donor pairs, a single minor mismatch may be of little biological significance. Molecular typing relies on polymerase chain reaction (PCR) amplification of specific gene segments and can be performed 1) at a level corresponding to the specificities identified by serology (low resolution), 2) at a level where a limited number of alleles are possible (intermediate resolution), or 3) at a level where the specific allele is identified (high resolution). Sequence-based HLA typing is the most precise technique available. Improvement in typing techniques through high-resolution molecular typing has had a profound impact in reducing the risks of immunologic complications with unrelated allogeneic transplantation.<sup>33</sup> This is highlighted by studies finding that just over 50% of donor-patient pairs typed by conventional serologic typing are found to be matched at the allele level when retrospectively typed using high-resolution techniques. This translated into higher probabilities of severe acute GVHD and transplant-related mortality, as well as lower survival in those retrospectively identified allele-mismatched patients.<sup>34</sup> The fact that immunologic complications (GVHD) often develop even among HLA genotypically identical siblings suggests that this system is not the only mediator of immunologic complications following allogeneic transplantation. In HLA-matched transplants, GVHD is felt to result from mismatching of poorly defined minor histocompatibility antigens. The only easily identified minor histocompatibility antigen is the HY antigen.<sup>35</sup> The chance of mismatching for these minor antigens should increase with increasing distance in the relationship between donor and recipient.<sup>36</sup>

### Donor Factors

Efforts to identify a donor is a lengthy process and must be carried out as soon as a patient has been referred, often before a definitive decision related to transplantation has been made. Usually all potential sibling donors are typed first and if no compatible donors are identified, an unrelated donor search is performed. Because of the shrinking size of households, the frequency of unrelated donor transplant is becoming more common and is currently estimated to account for one third of all allogeneic transplants.<sup>37</sup> To help facilitate this process, registries containing HLA-typed potential adult donors were created. The largest of these registries is the National Marrow Donor

Program (NMDP), which was established in 1986 to facilitate donor enrollment as well as the donor search process. There are more than 7.5 million individuals listed on these registries with approximately 800,000 new volunteers added annually.<sup>38</sup>

Since the early 1990s the use of PBPCs has become increasingly common and now constitutes the most common HPC source. The rationale for using PBPCs is derived from studies in the autologous setting that demonstrated accelerated recovery of hematopoiesis when compared to traditional marrow transplantation.<sup>39-40</sup> For donors, this has eliminated the risks and morbidity associated with general anesthesia and marrow harvest. Recombinant growth factors such as G-CSF are used to mobilize HPCs into the peripheral blood where they may be collected through one or more leukapheresis procedures. In addition to the benefits to the donor, PBPC transplantation offers a number of advantages to the recipient. G-CSF-mobilized PBPCs contain more HPCs than marrow grafts.<sup>41</sup> This has shortened the duration of absolute neutropenia and thrombocytopenia in recipients by approximately 4 to 8 days.<sup>41,42</sup> Donor T cells are found in much higher concentration in PBPC grafts and help to facilitate engraftment especially after less dose-intensive preparative regimens. The presence of higher numbers of T cells initially raised the concern for greater frequency and severity of GVHD; however, many large studies have shown that the risk of severe acute GVHD using PBPC grafts does not exceed the risk seen in traditional marrow grafts.<sup>43,44</sup> The incidence of chronic GVHD in patients receiving PBPC grafts has tended to be higher than those receiving marrow grafts. Thus, longer follow-up will be needed before it is safe to conclude that these two sources are comparable.<sup>45,46</sup>

Only a fraction of patients will have a fully matched unrelated donor identified despite the growing number of typed volunteers on worldwide registries. Populations of European ancestry have an approximately 50% chance of identifying a matched unrelated donor, whereas ethnic or mixed communities have as low as a 10% chance.<sup>47</sup> This disparity is largely the result of the increased polymorphisms with respect to HLA seen in certain minority groups.<sup>48</sup> For this reason, alternative donor transplants are an area of active research.

Umbilical cord blood (UCB) offers another source of hematopoietic stem cells for use in allogeneic transplantation. UCB transplants have increased the available donor pool because of the reduced need for HLA compatibility between the donor and recipient. In addition, UCB has shortened the time it takes to secure a donor, because the cells are readily available and do not require delays in the collection process. One review found that 19% of patients who did not have a potential HLA-matched unrelated donor were able to find a UCB unit matched at four of six HLA antigens. This same analysis found that the median time from search to clearance of an unrelated donor was 49 days; securing UCB units took just 13.5 days.<sup>49</sup> Because the number of cells required for successful engraftment is dependent on recipient size, a limiting factor for this form of transplant is the low number of HPCs in a unit of cord blood. Recent registry

analysis<sup>50</sup> evaluating outcomes for UCB transplantation in pediatric patients with leukemia found similar leukemia-free survival for one- or two-antigen mismatch UCB transplants when compared to HLA-matched unrelated transplants. This study found similar rates for acute GVHD between the mismatched UCB transplant and unrelated donor transplant recipients. Treatment-related mortality was higher in two-antigen mismatch UCB when compared to one-antigen UCB or matched unrelated donor recipients. In addition, patients who had a low-cell dose, one- or two-antigen mismatch UCB transplant had higher transplant-related mortality than those receiving a matched, unrelated donor transplant. Median time to neutrophil and platelet engraftment were higher following one- to two-antigen mismatched UCB transplants when compared to unrelated marrow recipients.<sup>50</sup>

Retrospective studies in adults have confirmed a low rate of acute GVHD when comparing one-antigen and two-antigen mismatch UCB transplants to one-antigen mismatch unrelated marrow recipients but most have demonstrated inferior outcomes when compared with matched unrelated donor recipients. These studies also confirmed a higher graft failure rate and treatment-related mortality for UCB transplants in adults when compared to matched unrelated donor transplants and lower leukemia-free survival.<sup>51</sup> Nonetheless, UCB transplants are a feasible option for adults who do not have a related or unrelated, matched donor and strategies for improving engraftment are an active area of research.

Haploidentical transplants offer another option when a matched sibling or unrelated donor cannot be identified. The advantage of these transplants is that parents, children, or half-matched siblings can serve as a donor. These forms of transplants call for highly selecting the collected product for CD34+ HPCs in an effort to minimize the amount of T cells in the final infusion. In this way, the risk for GVHD (which would otherwise be universal with transplants from haploidentical donors) is reduced. In addition, the conditioning regimen often includes antibodies directing against T cells. While these efforts reduce the risk for GHVD, delayed immune reconstitution occurs, resulting in a high risk for infectious complications.<sup>47,52</sup>

### Recipient Factors

It is estimated that over 20,000 allogeneic transplant procedures have been performed worldwide for a variety of malignant and nonmalignant disorders.<sup>53</sup> The International Bone Marrow Transplant Registry (IBMTR), an invaluable resource that collects data from more than 400 centers worldwide, allows transplant physicians to see current transplant trends, compare outcomes of different transplant strategies, and conduct retrospective and prospective studies in HSCT. According to data submitted to the IBMTR, the following trends have been noted: Currently the most common diagnoses for which allogeneic progenitor cell transplantation is being performed are acute myelogenous leukemia (AML), acute lymphocytic leukemia (ALL), chronic myelogenous leukemia (CML), and

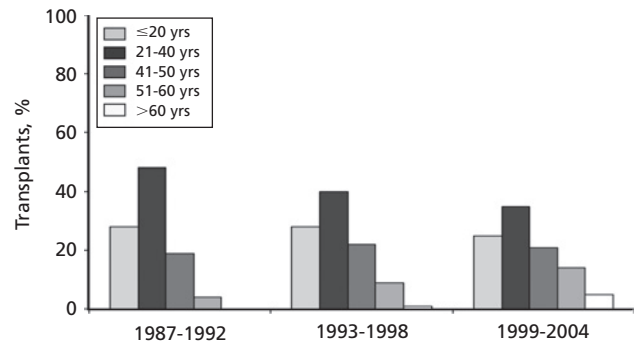


**Figure 35-1.** The proportion of older recipients of allogeneic transplants for AML, ALL, CML [as reported to the Center for International Blood and Marrow Transplant Research ([www.cibmtr.org](http://www.cibmtr.org))] is increasing.

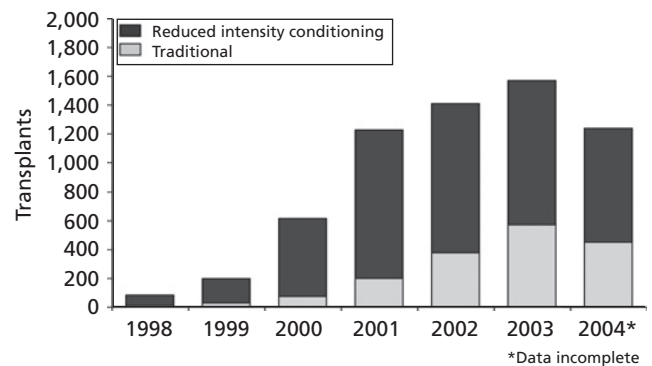


**Figure 35-2.** The proportion of allogeneic transplants performed using reduced-intensity conditioning is increasing [data incomplete for 2004; provided by the Center for International Blood and Marrow Transplant Research ([www.cibmtr.org](http://www.cibmtr.org))].

myelodysplastic syndromes (MDS) (in decreasing order). The indications for transplantation have changed over time; eg, CML is no longer the most commonly transplanted hematologic malignancy because of the advent of imatinib. Likewise, the average age of transplant recipients has increased over time. The proportion of patients receiving allografts over the age of 50 years is approaching 15%. Paralleling the increasing age of recipients has been the use of less-intense conditioning regimens (Figs 35-1 and 35-2).<sup>37</sup>

Numerous recipient factors determine the outcome following transplantation and include the disease stage at transplantation, recipient age, performance status, and presence of comorbidities. Apart from poor performance status, there is no one factor that precludes allografting in an individual patient. Instead, the decision to undertake transplantation depends on a careful assessment and discussion of the risks and benefits of the procedure when contrasted with the natural history of the disease. All potential transplant recipients undergo an extensive pretransplant evaluation that usually includes a complete history and physical examination in addition to evaluation of cardiac, pulmonary, hepatic, and renal function.



## Preparative Regimens

In allogeneic transplantation, the preparative regimen serves two main purposes: eradicating the underlying malignancy and inducing a state of immune tolerance to allow for the donor cells to engraft and expand. Generally speaking, efforts to decrease relapse through intensification of the preparative regimen have come at the expense of increased treatment-related mortality. As in autologous transplantation, there is no one standard preparative regimen in the setting of allogeneic transplantation. Allogeneic transplant conditioning regimens can generally be divided into chemotherapy-based protocols or total body irradiation (TBI)-based protocols.

TBI provides both immunosuppressive and myeloablative effects. The use of fractionated regimens and organ-shielding has lowered the toxicity.<sup>54-57</sup> Most modern regimens deliver a total dose of 1000 cGy to 1500 cGy using a variety of fractionation schedules. Although there is some evidence that further escalation of the total dose of TBI is more effective at preventing relapse, these benefits have been offset by increased nonrelapse mortality.<sup>58,59</sup> TBI is most often combined with high-dose cyclophosphamide (Cy), a potent immunosuppressive agent.<sup>60</sup> Other chemotherapy regimens with demonstrated efficacy in conjunction with TBI included etoposide either alone or together with Cy. Efficacy appears similar between these regimens with the combination of etoposide and Cy showing increased toxicity in patients with advanced disease.<sup>61,62</sup> TBI-based regimens are still commonly used in all disease states; however, it remains the most favored transplant regimen for patients with ALL (often combined with etoposide).<sup>63</sup> Although efficacious, TBI is associated with a number of short- and long-term complications including secondary malignancies, cataracts, and endocrine dysfunction.<sup>64</sup>

The creation of non-TBI-containing regimens developed out of a wish to decrease the above-mentioned long-term toxicities as well as expand this therapy to centers without dedicated radiotherapists. The most extensively studied regimen was one created by Santos in the early 1980s<sup>65</sup> and later modified by Tutschka.<sup>66</sup> Santos' original regimen replaced TBI with busulfan (Bu) at a dose of 16 mg/kg divided orally every 6 hours over 4 days combined with Cy 200 mg/kg divided in four daily doses (BuCy4). This regimen was found to be too toxic with a high rate of early transplant-related mortality and was later modified to the BuCy2 regimen with a decrease in the dose of Cy to 60 mg/kg for 2 days. This regimen remains in wide use today for various malignancies in both allogeneic and autologous transplantation. The safety and efficacy of the BuCy2 regimen when compared to TBI-Cy has been extensively evaluated in multiple, randomized studies.<sup>67-71</sup> Most of these studies showed comparable outcomes for these two regimens; however, a few demonstrated superiority of the TBI-containing regimens especially for those AML and ALL patients with advanced disease. With these exceptions there currently are no data that confirm the superiority of one specific regimen over another. Recent modifications to the BuCy2 regimen with pharmacokinetic (PK)-guided dosing of Bu and/or an intravenous (IV) formulation probably eliminate any small

advantage in the TBI-Cy regimen that has thus far been inconsistently demonstrated.

The dose-limiting side effect of oral Bu is hepatotoxicity with veno-occlusive disease (VOD) of the liver a major limitation of the BuCy2 regimen. It has been well documented that oral dosing of Bu results in frequent variability in inter- and inpatient steady-state drug concentrations.<sup>72-76</sup> Elevated levels of Bu in the blood have been shown to result in higher incidences of VOD of the liver,<sup>77</sup> while low doses may be associated with relapse (in CML)<sup>78</sup> and nonengraftment.<sup>79-82</sup> It has been hypothesized that by reducing this variability, results with Bu-containing regimens might be improved. PK-guided oral dosing followed by administration of an IV formulation of the drug has been evaluated.

The need for PK-guided Bu dosing is strongest in pediatric transplantation where the variability of oral dosing is most pronounced. This is especially true for patients less than 4 years of age who have increased clearance of the drug when administered orally because of a higher conjugation rate with glutathione in the hepatocyte.<sup>83</sup> Bolinger et al<sup>84</sup> performed one of the earliest trials evaluating targeted-dosed Bu in pediatric patients undergoing allogeneic transplantation. In this study, adjustment from standard dosing was required in a majority of the patients to achieve the target steady-state concentration. By so doing, 30 of 32 (94%) patients successfully achieved the target concentration. This strategy improved the rate of engraftment when compared to a historical group of patients who received standard-dosed Bu. Similar results were shown by Bleyzac et al<sup>85</sup> who were also able to document lower incidences of VOD of the liver when compared to historical controls. PK-guided strategies have also been shown to be feasible and result in low treatment-related mortality in adult patients receiving oral Bu regimens.<sup>86,87</sup>

In 1996, a patent was filed for an IV formulation of Bu. In 2000, researchers at M.D. Anderson Cancer Center performed a safety and feasibility study of the IV formulation and determined the bioequivalent dose.<sup>88</sup> Phase II studies performed with the IV formulation have determined a lower incidence of VOD of the liver, death from VOD, and treatment-related mortality when retrospectively compared to patients treated during the same period who received the standard oral BuCy2 regimen.<sup>89-93</sup> However, questions related to whether IV Bu is superior to oral Bu, whether PK-guided dosing of the IV formulation is needed, and whether oral PK-guided doses are equivalent to the IV formulation remain unanswered in the absence of randomized studies.

Additional modifications to the BuCy regimen have stemmed from reports determining that Cy and its metabolites contribute not only to the development of hemorrhagic cystitis after transplantation but also to the liver toxicity of the preparative regimen.<sup>94</sup> Therefore, fludarabine has replaced Cy in many modern myeloablative regimens. Initial studies with the combination of fludarabine and Bu have produced high engraftment rates with low levels of toxicity.<sup>95,96</sup>

## Reduced-Intensity Conditioning Regimens

Traditionally, allogeneic transplantation has been limited to younger patients because of toxicity related to the intensive

**Table 35-2.** Examples of Reduced-Intensity Conditioning Regimens

1. Total body irradiation at doses  $\leq 500$  cGy
2. Total busulfan dose  $\leq 9$  mg/kg
3. Total melphalan dose  $\leq 140$  mg/m<sup>2</sup>
4. Regimen includes a purine analog—fludarabine, cladribine, or pentostatin

**Table 35-3.** Most Commonly Used Reduced-Intensity Conditioning Regimens

1. Low-dose total body irradiation (200 cGy)  $\pm$  fludarabine
2. Busulfan + fludarabine  $\pm$  others
3. Fludarabine + cyclophosphamide  $\pm$  others
4. Fludarabine + melphalan  $\pm$  others
5. Fludarabine  $\pm$  others

conditioning regimens. Unfortunately, the cancers for which this therapy is most commonly prescribed occur in older adults. Reduced-intensity conditioning regimens employ lower doses of chemo- or radiotherapy to allow for engraftment and rely on the GVT effect to prevent relapse. As a result, patients who traditionally were felt to be too old for allogeneic transplant or who suffer comorbidities that previously would have precluded them can now safely undergo this therapy. There is no one standard reduced-intensity regimen, with commonly used regimens differing in the degree of intensity administered. Some regimens employ the same agents used in full-intensity regimens, but at reduced doses. On the other end of the spectrum are regimens that provide minimal cytotoxic agents and instead rely on immunosuppressive treatments to allow donor engraftment. Agreed-upon definitions of reduced-intensity regimens as well as commonly employed agents used are listed in Tables 35-2<sup>97</sup> and 35-3.<sup>98</sup>

### Potential Complications of Dose-Intensive Therapy Followed by HSCT

The potential risks resulting from high-dose chemoradiotherapy and blood and marrow transplantation are numerous and include toxicity from the preparative regimen, infections resulting from granulocytopenia or posttransplant immunodeficiency, hemorrhage caused by thrombocytopenia and tissue toxicity, and, in the case of allogeneic transplants, transplant rejection and GVHD.

The creation of better supportive medications has minimized many of the acute toxicities associated with high-dose chemoradiotherapy regimens. This includes the incorporation of selective type three 5-hydroxytryptamine (5-HT<sub>3</sub>) receptor antagonists, which has decreased the incidence of severe treatment-related nausea.<sup>99</sup> The addition of palifermin (recombinant human keratinocyte growth factor) and mesna (2-mercaptoethane sodium sulfonate) have been shown to reduce the incidence of severe radiation-induced mucositis<sup>100</sup>

and chemotherapy-induced cystitis,<sup>101</sup> respectively. The risk for serious organ toxicities and infections are detailed below. Generally accepted mortality rates associated with autologous transplants are approximately 5%, whereas allogeneic transplant recipients have a 10% to 50% risk for transplant-related death depending on host factors, including age, comorbidities, disease stage, baseline toxicities, and donor factors (namely, matching).

### Myelosuppression

Shortly after infusion, HPCs migrate to sites in the lungs, liver, spleen, and marrow. For most patients, some degree of marrow cellularity can be demonstrated within 14 days of transplantation. Neutrophil and platelet engraftment is defined as being the first of three consecutive days in which the absolute neutrophil count is  $>500/\mu\text{L}$  and the platelet count is  $>20,000/\mu\text{L}$  (without transfusion support), respectively. This generally occurs 14 to 24 days after stem cell infusion, with longer time to engraftment in cord blood transplants. Before engraftment, patients require aggressive hematologic support with transfusion of platelets and red cells. Blood components must be irradiated to minimize the possibility of graft-vs-host reactions mediated by blood donor T cells.<sup>102</sup> For patients experiencing prolonged cytopenias, growth factors may be used to shorten the duration of aplasia without increasing the risk of GVHD or relapse.<sup>103,104</sup> In the allogeneic setting, engraftment of donor cells can be documented by use of molecular tests such as DNA restriction fragment length polymorphisms, or by PCR of microsatellite regions demonstrating a donor pattern. Following successful transplantation, cells of the recipient's reconstituted hematologic and immunologic systems are derived primarily from the donor's blood or marrow, although in some cases mixed chimerism occurs in which both donor- and recipient-derived cells are present in the recipient's circulation and marrow.<sup>105</sup>

### Graft Failure

The failure to recover hematologic function or the loss of marrow function after initial reconstitution constitutes graft failure. Graft failure is an unusual event for autologous transplant recipients but can occur in 5% to 11% of HLA-identical allogeneic recipients.<sup>106</sup> Graft failure generally takes place within 60 days of transplantation although late graft failure has been known to occur.<sup>107</sup> Several factors are known to increase the risk of graft failure in allogeneic transplantation, including a low nucleated cell count infused, increasing HLA disparity between donor and host, T-cell depleted grafts, and inadequate immunosuppression of the host. Historical graft failure rates were as high as 35% in patients undergoing transplantation for aplastic anemia, which was felt to be caused in large part by alloimmunization from prior transfusions. This has now been reduced to  $<10\%$  for patients with aplastic anemia through the minimizing of pre-transplant transfusions and administration of leukocyte-reduced, irradiated blood components.<sup>108</sup> Evidence now exists implicating host T cells as the mediators of an active host immune response against minor alloantigens expressed by the donor cells. On the

basis of this understanding, several strategies to further eliminate or inactivate host T cells have evolved, including the use of additional or increased intensity immunosuppressive agents such as fludarabine or the elimination of host T cells through the use of antithymocyte globulin (ATG) or anti-CD52.<sup>109-111</sup> Treatment of graft failure depends on whether donor cells can be detected in the patient's marrow. If present, a repeat infusion of donor cells can be attempted with or without further conditioning. When no donor hematopoietic cells are detected, a second conditioning regimen may be administered followed by donor stem cells; however, outcomes in this setting are extremely poor.<sup>112</sup>

Graft-vs-Host Disease

GVHD represents one of the most frequent complications of allogeneic transplantation and has been the major barrier to wide-scale application of this therapy. GVHD results from the recognition of host tissues as foreign by donor immunocompetent cells. The incidence increases with greater HLA disparity between the donor and host. A fundamental problem for allogeneic transplantation is the close association between this complication and the derived benefit resulting from a GVT effect. Both GVHD and GVT effect are mediated by donor T cells with both being reduced when T cells are depleted from the infused graft (referred to as T-cell-depleted transplants). Identifying and separating the target antigens resulting in GVHD and GVT effect are active areas of research that may one day result in maximizing the therapeutic potential of this modality while eliminating this frequent obstacle.<sup>113</sup>

Two forms of GVHD are commonly distinguished: acute and chronic. Historically, acute GVHD was defined as occurring within the first 100 days of transplant and chronic GVHD was defined as occurring after the 100-day mark. It is now clear that this time point was an artificial one and clinical manifestation and histologic findings are now the sole factors used in defining these distinct entities.<sup>114</sup>

The incidence of acute GVHD varies from 20% to 70%, depending upon the intensity of the conditioning regimen, the extent of histocompatibility differences with the donor, the age of the recipient, and the stage of primary disease.<sup>115-120</sup> Acute GVHD is graded clinically using a standardized system that takes into account clinical changes in the primarily affected organs: skin, liver, and gastrointestinal tract. This grading system allows for quantitative estimates of disease severity and response to therapy with scoring consisting of Grades I to IV (Table 35-4).<sup>121</sup> These clinical findings are usually confirmed by pathologic changes in the affected organs, although the pathologic findings do not change the grading or staging of the disease. Corticosteroids remain the standard initial treatment, but even with prompt initiation of such therapy, the treatment is suboptimal. Fewer than 50% of patients with acute GVHD have a durable response after initial therapy; most patients require secondary treatment.<sup>122,123</sup> Attempts to improve the complete response rate by adding additional therapies to corticosteroids or by using higher doses have been unsuccessful.<sup>124,125</sup> Moreover, the outcome for patients with steroid-refractory GVHD is poor, with a mortality rate of 70%; at this time, no therapy has been shown to affect survival.<sup>126</sup> The causes of death among patients with advanced GVHD include organ failure and infections related to poor immune reconstitution. For these reasons, improving prophylaxis has been the preferred strategy.

The prophylactic use of cyclosporine (CSA) and methotrexate (MTX) are effective prophylaxis for acute GVHD and have been shown to improve survival.<sup>115</sup> Corticosteroids have also been successfully used in combination with CSA for GVHD prophylaxis; however, the addition of prednisone to CSA and MTX has been shown not to add additional benefit.<sup>127</sup>

CSA is a cyclic polypeptide that prevents T-cell activation by inhibiting interleukin-2 production and expression. Although effective as GVHD prophylaxis, CSA has significant toxicities including hypertension, nephrotoxicity, hypomagnesemia, risks

Table 35-4. Organ Stages of Acute GVHD

| Organ Stage | Organ                                                            |                           |                                                                                      |
|-------------|------------------------------------------------------------------|---------------------------|--------------------------------------------------------------------------------------|
|             | Skin (body surface area)                                         | Liver (mg/dL bilirubin)   | Gastrointestinal Tract (24-hour stool output)                                        |
| 0           | No rash                                                          | <2.0                      | <500 mL or <280 mL/m <sup>2</sup>                                                    |
| I           | Rash <25% or 25% to <50%                                         | <2.0                      | <500 mL or <280 mL/m <sup>2</sup>                                                    |
| II          | ≥50% or generalized erythroderma                                 | 2.1 to 3.0                | 501 to 1000 mL or 280 to 555 mL/m <sup>2</sup> or persistent nausea and/or vomiting  |
| III         |                                                                  | 3.1 to 6.0 or 6.1 to 15.0 | 1001 to 1500 mL or 556 to 833 mL/m <sup>2</sup> or >1501 mL or 833 mL/m <sup>2</sup> |
| IV          | Generalized erythroderma with bullous formation and desquamation | >15.1                     | >1501 mL or 833 mL/m <sup>2</sup> with severe abdominal pain and ileus               |

for seizures, hypertrichosis, gingival hyperplasia, tremors, and anorexia.<sup>128</sup> Typically CSA is initiated intravenously 1 to 2 days before stem cell infusion and converted to oral dosing when possible. The risk of acute GVHD increases when cyclosporine concentrations in blood drop below a target level.<sup>129</sup>

Tacrolimus is a macrolide lactone that closely resembles CSA in mechanism of action, spectrum of toxicities, and pharmacologic interactions. In a randomized study,<sup>119</sup> the prophylactic regimen of tacrolimus and MTX was demonstrated to reduce Grade II-IV acute GVHD when compared to CSA and MTX. However, there was a higher regimen-related death rate in patients with advanced disease who received tacrolimus in this study, possibly related to the use of tacrolimus at levels beyond those currently recommended. The implications of this finding are unclear and generally CSA and tacrolimus are viewed as equivalent. The addition of MTX has the potential for increased severity of regimen-related mucositis and delays in engraftment. At M.D. Anderson Cancer Center, a modification of the tacrolimus-MTX regimen using “mini-dose MTX” was found to be as effective as full dose therapy with less toxicity (Fig 35-3).<sup>130</sup>

ATG has also been used effectively in the prophylactic setting.<sup>131</sup> In patients with aplastic anemia who underwent transplantation using a Cy and ATG conditioning regimen, low rates of acute GVHD (15%) and chronic GVHD (34%) were reported and a significant survival benefit at 3 years was identified for patients receiving this regimen when compared with historical controls who received Cy alone (92% vs 72%).<sup>109</sup>

In addition to the immunosuppressive agents above, T-cell depletion before marrow infusion has been shown to effectively reduce the incidence and severity of GVHD; however, it also results in increased graft rejection, infectious complications, and relapse rates in a disease-specific manner.<sup>8</sup> Selective T-cell subset depletion or “add-back” of allo-depleted T cells are future strategies that may be effective for reducing the risk of acute GVHD while preserving the GVT effect.<sup>52,132</sup>

Chronic GVHD shares many features with various autoimmune disorders, including autoantibodies and disease

manifestations that are similar to such disorders as the sicca syndrome and scleroderma. The manifestations of chronic GVHD are extensive with nearly every organ potentially affected.<sup>114</sup> The consequences for affected patients can be profound, with its presence or absence being the most significant determinant of long-term outcome and quality of life following allogeneic HSCT.<sup>133</sup> Approximately 60% to 70% of patients who receive an allogeneic transplant are affected to some degree by chronic GVHD.<sup>134</sup> Despite progress in other areas of this field, our understanding of this entity is limited; therefore, advancements in the prevention and treatment of chronic GVHD have been sparse.

## Organ Toxicity

### Veno-Occlusive Disease of the Liver

VOD, also referred to as sinusoidal obstruction syndrome, is a complication of both allogeneic and autologous transplant as well as occasionally chemotherapy regimens in the nontransplant setting. The process occurs as a result of direct injury to the hepatic venous endothelium. The syndrome is characterized by the constellation of signs and symptoms consisting of tender hepatomegaly, jaundice, weight gain, and ascites. The incidence of this syndrome has varied among reports and has ranged from 5% to 50% of all allogeneic and autologous transplants.<sup>135,136</sup> These studies suggest VOD is life-threatening in roughly one-quarter of cases. Risk factors include preexisting liver disease, high-intensity conditioning regimens, prior chemotherapy regimens, and mismatched or unrelated donor transplants.<sup>136</sup> Preliminary reports have found defibrotide (a polydeoxyribonucleotide) to be a promising treatment for patients suffering from severe VOD.<sup>137</sup>

### Lung

Myriad pulmonary complications can occur in patients following autologous and allogeneic transplantation. Most of these complications are a result of acute or delayed complications of the conditioning regimen and previous treatments. It is important to distinguish these entities from an infectious etiology. Direct toxicity can result from the conditioning regimen including carmustine-induced

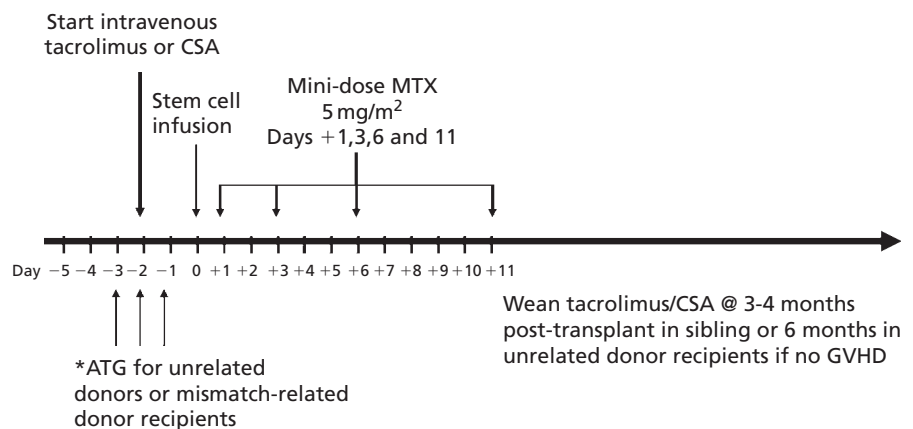


**Figure 35-3.** Standard GVHD prophylaxis at M.D. Anderson Cancer Center. CSA = cyclosporin A; ATG = antithymocyte globulin; GVHD = graft-vs-host disease.

pulmonary toxicity and radiation-induced lung injury. Diffuse alveolar hemorrhage (DAH) may occur in the immediate post-transplant period during pancytopenia. One study found a strong association between DAH and previous thoracic irradiation, whereas no association was found between the presence of thrombocytopenia, coagulopathy, or neutropenia.<sup>138</sup> Idiopathic pneumonia syndrome may occur following allogeneic transplant. It is described as a diffuse, non-infectious pneumonia that in animal models appears to be an immune-mediated process in part induced by the toxicity of the conditioning regimen.<sup>139,140</sup> Bronchiolitis obliterans is a pulmonary manifestation of chronic GVHD.<sup>114</sup>

## Infections

Patients undergoing autologous and allogeneic transplants are at risk for a variety of infections as a result of the myelosuppression induced by the chemo- and radiotherapies employed during conditioning, delayed immune reconstitution following myeloablative therapy, and, in the case of allogeneic transplantation, immunosuppression used to prevent and treat GVHD as well as GVHD itself. Risk for infection is generally divided into three phases: Phase I, the period following preparative regimen while awaiting engraftment; Phase II, following engraftment and up to Day 100; and Phase III, from Day 100 to months and year(s) following transplantation. Infectious risks for each of the three periods varies, respectively, depending on the degree of tissue toxicity and length of neutropenia following the preparative period, the presence of acute GVHD, and the presence of chronic GVHD. Based on the high risk and associated morbidity resulting from bacterial, fungal, and viral pathogens, several prophylactic and surveillance strategies have been developed for patients undergoing transplantation.

Bacterial, candidal, and herpes simplex virus (HSV) infections pose the greatest risk for patients during the preengraftment period. In addition to conditioning-induced myelosuppression, patients often have impaired primary immunity as a result of indwelling catheters and tissue toxicity (mucositis, gastroenteritis, etc). Bacterial prophylaxis with a quinolone antibiotic is often employed in an effort to suppress intestinal flora and prevent serious gram-negative infections<sup>141,142</sup>; however, this is controversial and currently not recommended in guidelines from the Infectious Disease Society of America (IDSA)/Centers for Disease Control and Prevention (CDC).<sup>143</sup> The prompt administration of broad-spectrum antibiotics following a neutropenic febrile event is of paramount importance in preventing morbidity of bacterial infections.

In a randomized study,<sup>144</sup> the prophylactic use of fluconazole has been shown to reduce the incidence of systemic and superficial fungal infections. Fluconazole has not, however, been shown to affect the incidence of infections with resistant organisms such as *Candida krusei* or *C. glabrata* and invasive mold infections. Prophylaxis against other invasive fungal infections such as *Aspergillus* species is an area of debate with some studies supporting the use of newer azole agents<sup>145</sup> or echinocandins.<sup>146</sup>

Herpes simplex virus reactivation is seen commonly in seropositive patients during the first 6 weeks after transplantation unless prophylaxis is administered. Acyclovir prophylaxis has been

shown to prevent reactivation, reduce morbidity, and lower the incidence of antibiotic resistance and is routinely administered.<sup>147</sup> Patients in whom HSV reactivation occurs should be treated with acyclovir or valacyclovir. For patients who develop resistance after multiple episodes of HSV reactivation, foscarnet may be required.<sup>148</sup>

Prophylaxis against *Pneumocystis carinii* (PCP) is recommended following engraftment and continued for a minimum of 6 months following transplantation or while taking immunosuppressive drugs. Trimethoprim-sulfamethoxazole is the agent of choice for PCP prophylaxis given that it has the lowest breakthrough rate. Various other agents have been used including dapsone and aerosolized pentamidine.<sup>149</sup>

Cytomegalovirus (CMV) infections were once a common and fatal occurrence in the first 3 months following marrow transplantation. The routine prophylactic use of ganciclovir has largely eliminated CMV disease after transplantation. Randomized studies have clearly demonstrated efficacy in reducing the incidence of CMV disease by the prophylactic treatment of all CMV-seropositive patients.<sup>150</sup> However, this strategy is problematic in that many patients will require discontinuation of therapy because of the development of neutropenia, which places the patients at increased risk for other infections. The creation of highly sensitive tests capable of detecting CMV reactivation before overt CMV disease has allowed for an alternative strategy to routine prophylaxis. These tests include direct detection of CMV pp65 antigen in peripheral blood leukocytes<sup>151</sup> and detection of CMV DNA in leukocytes, plasma, or serum.<sup>152</sup> Preemptive therapy when reactivation is first detected has been shown to result in similar rates of CMV-related death and survival when compared with universal ganciclovir prophylaxis starting at the time of engraftment. Patients receiving routine prophylaxis experienced less CMV disease in the first 100 days after transplantation; however, this benefit was offset by a higher rate of invasive fungal infections and late CMV disease.<sup>151</sup> Thus, current recommendations support either approach for patients at risk for CMV disease (ie, all CMV-seropositive recipients and seronegative recipients with seropositive donors). For CMV-seronegative patients with CMV-seronegative donors, the use of CMV-seronegative or leukocyte-reduced blood components are effective in preventing primary infections.<sup>153</sup>

Viral infections continue to pose significant challenges to clinicians managing posttransplant patients. In addition to community respiratory viruses, intestinal viruses are easily transmitted through person-to-person contact and have been associated with increased morbidity and mortality. Because of a lack of specific therapies for these agents, the focus must be on appropriate isolation of patients and the prevention of person-to-person transmission.

## Long-Term Complications of High-Dose Therapies

Several long-term complications occur following HSCT, requiring patients to maintain regular office visits and practitioners familiar with posttransplant follow-up guidelines to monitor patient progress. Regular eye screening for cataracts is required especially for those who received a TBI-containing regimen or steroids for

the treatment of GVHD.<sup>154</sup> Several endocrine complications occur including hypothyroidism, stunted growth and development (in pediatric patients), and premature gonad failure/sterility.<sup>155</sup> Patients who have undergone autologous HSCT are at increased risk for treatment-related MDS and AML, especially if they were subject to prior radiation (especially to the pelvis), were heavily pretreated, were slow PBPC mobilizers, or were subject to TBI-containing conditioning regimens.<sup>156</sup> Both autologous and allograft recipients are at higher risk for additional secondary cancers especially bone, head, neck, and connective tissue malignancies.<sup>157</sup>

## Disease Indications

### Acute Myelogenous Leukemia and Myelodysplastic Syndromes

AML and MDS currently constitute the most common indication for allogeneic transplantation. Treatment decisions pertaining to postinduction therapy are centered on disease risk for relapse as determined by cytogenetic abnormalities.<sup>158</sup> Most would agree that suitable candidates with intermediate risk and all poor-risk candidates who have a suitable donor should undergo allogeneic transplantation while in first complete remission (CR).<sup>159</sup> Patients who are in first relapse/second remission should be offered an allogeneic transplant regardless of their original cytogenetics provided that they have a suitable donor. Although the likelihood of success with allogeneic transplantation is substantially less for this group of patients when compared to those in first CR, no other therapy is known to offer long-term cures.<sup>160</sup> Reduced-intensity conditioning regimens have allowed for allogeneic transplantation to be offered to older adults with AML and MDS with acceptable treatment-related mortality.<sup>161</sup> This is extremely pertinent for disease that has an average age of onset in the seventh decade of life.

### Acute Lymphocytic Leukemia

Conventional chemotherapy offers prolonged remission and even a cure for most children and many adults. Accordingly, transplantation is not usually undertaken until after relapse.<sup>162</sup> First remission transplantation has been reserved for adults with high-risk disease such as patients with t(4:15) or t(9:22) cytogenetics.<sup>163</sup> Successful results of marrow transplants in first remission for these patients range from 30% to 60%, depending on patient age and donor type. The GVL effect may be less in ALL than is observed in CML or more indolent malignancies.<sup>164</sup> The rarity of allogeneic donor lymphocyte infusion inducing remission for relapses after allogeneic transplantation supports this observation. Patients with active leptomeningeal leukemia should have this complication treated before transplantation, because few marrow transplant preparative regimens have agents that cross the blood-brain barrier.

### Chronic Myelogenous Leukemia

Before the advent of tyrosine kinase inhibitors such as imatinib, CML represented the most common disease group treated with

allogeneic transplantation. A meta-analysis of four randomized trials of allogeneic transplantation in patients with CML in chronic phase found 10-year survival estimate of more than 60%.<sup>69</sup> Patients with favorable prognostic factors (young age, favorable donor characteristics, and newly diagnosed disease) have a demonstrated overall survival of over 70%, albeit with a significant risk for acute and chronic GVHD.<sup>165</sup> These results need to be viewed in the context of the promising Phase II trials with imatinib demonstrating a complete hematologic response in 95% and a major cytogenetic response in 65% of patients treated in chronic phase with a median follow-up of 4 years with minimal toxicity.<sup>166</sup> For this reason, expert consensus opinion has recommended allogeneic transplantation be limited to patients who have not achieved a prompt complete hematologic response with initiation of imatinib or have evidence of persistent cytogenetic abnormalities. There is not enough evidence to determine whether newer, more potent tyrosine inhibitors will change these recommendations.<sup>167</sup>

### Multiple Myeloma

Multiple myeloma currently represents the most common indication for autologous HSCT. This is based upon multiple randomized studies demonstrating higher complete remission rates, event-free survival, and overall survival in patients undergoing high-dose therapy followed by autologous HSCT when compared to chemotherapy alone. A French study<sup>168</sup> reported a CR and 5-year survival rates of 22% and 52% in the high-dose group vs 5% and 12% in the standard-dose study arm. Randomized studies have established melphalan 200 mg/m<sup>2</sup> as the “standard” regimen for patients under the age of 65.<sup>169</sup> The role of double autologous transplants was investigated in a protocol of the Intergroupe Francophone du Myelome. Attal et al<sup>170</sup> randomly assigned 399 untreated patients younger than 60 years to receive melphalan 140 mg/m<sup>2</sup> with TBI 8 Gy (single transplant) or melphalan 140 mg/m<sup>2</sup> followed by a second transplant using the TBI/melphalan regimen.<sup>170</sup> Response rate was similar, but overall and event-free survival was significantly improved in the double autologous transplant study arm. The subset of patients who did not achieve at least a partial remission with the first procedure appeared to benefit the most with this strategy. It should be noted, however, that all studies that determined the key role of autologous transplantation in this disease were conducted before the development of new “target” therapies, which may change radically the way multiple myeloma is treated in the future. To date, no therapy is felt to be curative for this disease. Recently, autologous stem cell transplant followed by reduced-intensity conditioning allogeneic transplantation in patients with a sibling donor has been explored with some randomized studies suggesting improvement in survival over tandem autologous transplant.<sup>171</sup>

### Lymphoma and Chronic Lymphocytic Leukemia

High-dose therapy followed by autologous HSCT is considered standard treatment for patients with diffuse large-cell lymphoma and Hodgkin disease who fail to achieve remission

following initial therapy, or subsequently relapse after an initial remission with chemo-sensitive disease. The Parma study was a randomized trial comparing standard salvage chemotherapy in first relapse for large-cell lymphoma to standard chemotherapy plus autologous marrow transplant.<sup>172</sup> This trial demonstrated a definitive advantage for patients receiving an autologous transplant. Autologous HSCT is the accepted treatment for patients with chemo-sensitive relapsed or primary refractory Hodgkin disease.<sup>173</sup> Patients who achieve a second CR following salvage treatment have the best outcomes with this approach with over 50% of patients achieving a long-term cure. There have been no randomized studies comparing TBI-containing to chemotherapy-alone conditioning regimens, but generally radiation-based regimens are avoided in those patients who have previously received radiation therapy. Recently, the incorporation of the CD20 monoclonal antibody, rituximab, into chemomobilization and conditioning regimens have been shown to improve outcomes.<sup>23</sup> The incorporation of radiolabeled antibodies into conditioning regimens is being explored.<sup>24</sup>

Allogeneic transplantation has been explored in patients with large-cell lymphoma and Hodgkin disease who have failed to achieve a suitable response to salvage chemotherapy or who have relapsed following an autologous HSCT with variable success. Treatment-related mortality has been high with full-intensity regimens; however, reduced-intensity regimens have been better tolerated and offer hope for cure in select patients.<sup>174,175</sup> In addition, encouraging results have been reported with reduced-intensity allogeneic transplants with indolent lymphomas and CLL.<sup>176,177</sup>

### Breast Cancer

Breast cancer once constituted the most common indication for high-dose chemotherapy followed by autologous HSCT. More recently, a series of randomized trials failed to demonstrate an improvement in long-term progression-free or overall survival in patients with high-risk<sup>178,179</sup> or metastatic breast cancer.<sup>180</sup> Apart from participation in a clinical trial, this approach is no longer justified.

### Future Directions

Fifty years have passed since Thomas and colleagues' first attempts at allogeneic HSCT. The field has advanced so that this therapy can now be offered to more patients with less risk for acute and long-term toxicity. Despite these advances, the challenge remains the discovery of new strategies that will improve disease-free survival without associated treatment-related mortality. Autologous and allogeneic HSCT remain curative for a minority of patients with advanced, refractory disease. Broadly applied, dose-intensification has not been able to significantly affect outcomes for this group of patients because of the overlap between toxicity and efficacy. Undoubtedly tomorrow's success will come through the incorporation of "targeted"

therapies that offer the hope of decreasing relapse without adding additional toxicity. In addition, new strategies for harnessing the immunotherapeutic potential of a GVL effect while reducing the risk GVHD will make allogeneic transplantation a more widely applied modality.

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### Disclaimer

The authors have disclosed no conflicts of interest.

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# Umbilical Cord Blood: A Reliable Source of Stem and Progenitor Cells for Human Transplantation

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The first successful allogeneic hematopoietic stem cell transplantation was performed in 1968 by Dr. Robert Good. The patient, who had severe combined immunodeficiency (SCID), received marrow from a sibling as the source of stem cells. Since then, thousands of patients have been cured of life-threatening hematologic disorders—malignant and nonmalignant—establishing the field of marrow transplantation. Chapter 35 provides an overview of allogeneic transplantation with an emphasis on the traditional sources of hematopoietic stem cells—namely, marrow and peripheral blood. Over the years, the National Marrow Donor Program (NMDP) in the United States and registries in other parts of the world have recruited, typed, and maintained databases on millions of voluntary unrelated adult donors. Despite the large number of registered donors, approximately two-thirds of the patients in need of a transplant are still unable to find a suitable marrow donor in a timely manner. This problem is even more of a concern for patients belonging to ethnic minorities. The need to breach the HLA barrier—to be able to perform transplants with partially HLA-mismatched donors—was realized by early investigators. However, the outcomes of partially mismatched hematopoietic stem cell transplants from marrow or mobilized peripheral blood, with or without T-cell depletion, have been suboptimal. This is mainly because of a higher incidence of graft failure, severe graft-vs-host-disease (GVHD), and poor immune reconstitution, leading to increased infection-related mortality. There has been a desperate need for stem cell sources that could result in adequate engraftment with acceptable rates of GVHD in the partially mismatched transplant setting.

The first part of this chapter focuses on the use of umbilical cord blood (UCB) as one such alternative source of hematopoietic stem and progenitor cells and describes the rapid emergence of its role in clinical hematopoietic transplantation. Recent research suggests that UCB applications may well extend beyond

the realm of hematopoietic reconstitution, with tremendous potential for the field of regenerative medicine. The latter part of the chapter provides an overview of the cord blood banking system.

## Cord Blood: Biology and Transplantation

The idea of using cord blood for hematopoietic transplantation arose from interactions among a group of physicians and scientists in the early 1980s. Using blood from near-term mouse donors, Dr. Ted Boyse at Memorial Sloan Kettering Cancer Center demonstrated reconstitution of hematopoiesis in lethally irradiated mice. In parallel, Dr. Hal Broxmeyer in New York and later in Indiana, performed early experiments characterizing the hematopoietic stem and progenitor cells in human UCB. He demonstrated that cord blood was a rich source of these cells, that they had higher proliferative capacity, and also that they could be readily cryopreserved.<sup>1</sup>

Hematopoietic stem and progenitor cells derived from cord blood are biologically different from those derived from marrow or mobilized peripheral blood, as evidenced by a large amount of data that has emerged from several groups studying stem cell biology. In-vitro studies of the sources show both quantitative differences, ie, a higher frequency of more primitive hematopoietic progenitor cells in UCB than in marrow, and functional differences, ie, a higher replating potential of UCB-derived colonies on semisolid cultures than of those derived from marrow. In-vivo studies show similar differences. Nonobese diabetic (NOD)/SCID repopulating cell assays indirectly measure the self-renewal and proliferative capacities of stem cells. Experiments have shown that the SCID-repopulating cell (SRC) frequency in UCB is three-fold higher than in marrow and sixfold higher than in mobilized peripheral blood, suggesting that UCB contains a higher proportion of primitive hematopoietic stem and progenitor cells.<sup>2</sup>

It is important to distinguish the hematopoietic stem and progenitor cells derived from cord blood and/or marrow from “embryonic stem cells.” Embryonic stem cells are *pluripotent* or

*totipotent* cells (cells capable of giving rise to all three basic germ layers—ectoderm, endoderm, and mesoderm) derived from the inner cell mass of the early embryo. With continued natural development of the embryo, the embryonic stem cells become more and more lineage committed, thus giving rise to cells of mostly one germ layer, but sometimes two. Such development results in a hierarchy of more restricted stem cells. Terms such as *multipotent*, *tissue restricted*, *adult*, or *somatic* stem cells are applied to these more committed stem cells. For example, liver stem cells give rise to hepatocytes; muscle stem cells give rise to muscle; and the hematopoietic stem or progenitor cells give rise to all three elements of blood—erythrocytes, leukocytes, and platelets. Marrow, as well as UCB, is rich in these more lineage-restricted multipotent hematopoietic cells, with some differences as alluded to above.

Pluripotent embryonic stem cells, at the current level of development, are not yet in clinical use. One reason is the development of teratomas in animal models. Clearly, these cells are highly proliferative and further studies are needed before they are ready for clinical application. Some investigators have raised concerns regarding epigenetic developmental abnormalities. Human egg harvesting is an invasive procedure. Not only are human eggs difficult to obtain, but ethical issues and religious beliefs make this an extremely controversial topic, because it involves the loss of human embryos. Therefore, despite their enormous potential, clinical therapeutic use of embryonic stem cells is unlikely to become a reality in the near future.

Conversely, marrow has been successfully used for hematopoietic transplantation for more than three decades. Furthermore, mobilized peripheral blood has been used, and now cord blood is increasingly being used as a cell source for transplantation. Taken together, these studies imply two important points: 1) that cord blood is a rich source of hematopoietic stem cells and 2) that there is a preponderance of more primitive types of stem cells in cord blood as compared to marrow or peripheral blood.

### **First Umbilical Cord Blood Transplantation: Proof of Principle**

In 1988, the use of cord blood as a source of hematopoietic stem cells was reported following an elaborate international, multi-institutional collaboration, when a 6-year-old boy from the United States with Fanconi anemia underwent successful transplantation in Paris.<sup>3</sup> The source of the donor cells was HLA-identical UCB from his unaffected sibling. Twenty years after the procedure, he is durably engrafted, immunologically competent, healthy, and cured of his hematologic condition. This outcome proved that cord blood harvested from a single donor contained a sufficient dose of stem cells for successful reconstitution of a pediatric patient's aplastic marrow.

### **Matched Sibling Donor UCB Transplantation**

This success led to an increasing number of matched related cord blood transplant procedures in the following years (~60 transplants over the next 5 years). Apart from demonstrating

acceptable rates of engraftment, it became clear from this early experience that among matched related donor transplants, cord blood caused less GVHD as compared to marrow, and that this property of cord blood might allow transplantation across genetic/HLA barriers. In 1992, the first public unrelated cord blood bank was established at the New York Blood Center by Dr. Pablo Rubinstein to explore the use of banked UCB from unrelated donors for hematopoietic stem cell transplantation.<sup>4</sup>

### **Unrelated-Donor UCB Transplantation**

In 1993, the first unrelated UCB transplantation was performed in a 3-year-old child with recurrent T-cell acute lymphoblastic leukemia. The outcomes of 25 successive transplants with unrelated UCB banked at the New York Blood Center, and transplanted at Duke University, were reported in 1996.<sup>5</sup> Important observations in these patients and subsequent reports from other centers and registries including the New York Blood Center and the European Cord Blood Registry (Eurocord)<sup>6-8</sup> demonstrated the following:

1. Unrelated cord blood could engraft in the marrow of children undergoing myeloablative therapy for leukemias and genetic diseases.
2. Reasonable outcomes could be achieved using partially HLA mismatched grafts.
3. The incidence and severity of acute and chronic GVHD was lower and milder than that seen with matched unrelated marrow transplants.
4. There was preservation of graft-vs-leukemia effects.<sup>9</sup>
5. Cell dose strongly correlated with clinical outcomes including time to engraftment and probability of overall engraftment and survival.
6. Engraftment times were observed to be slower than those of marrow or mobilized peripheral blood.

### **Prospective Study: COBLT**

Cord blood banking efforts in the United States were stimulated by the National Heart, Lung, and Blood Institute, which funded a prospective multicenter clinical trial of unrelated cord blood transplantation—the Cord Blood Transplantation Study (COBLT)—from 1997 to 2004. Three unrelated cord blood banks were established with funding provided through this study and all three banks established and followed common quality standards and standard operating procedures (SOPs). These SOPs addressed donor recruiting and screening. For screening, donors were evaluated for events in their medical history that would exclude them as donors (eg, multiple pregnancy, prematurity, placental deformity, self or a sibling diagnosed with cancer, prior receipt of a transplant, or demonstration of high risk behaviors likely to increase the chance of infection with blood-borne infectious diseases). SOPs were also established and validated for obtaining donor consent; obtaining medical histories; obtaining blood samples from maternal donors; cord blood collecting, processing, testing, cryopreservation, and storage; as well as searching for and releasing cord blood units for transplantation.<sup>10</sup>

Twenty-six transplant centers participated in this prospective clinical trial designed to examine the safety and efficacy of unrelated cord blood transplantation in infants, children, and adults with malignancies; children with congenital immunodeficiency disorders; and children with inborn errors of metabolism. The study participants employed common preparative regimens, prophylaxis against GVHD, and supportive care measures. Results in children with malignant and nonmalignant conditions were favorable, with 55% survival in children with malignancies and 78% survival in children with nonmalignant conditions. Results in a very high-risk group of adults were inferior to those seen in children and in individuals receiving marrow from an unrelated donor. Subsequent studies in adults, reported by single centers or registries, revealed more encouraging results.<sup>11,12</sup> The cumulative incidence of engraftment by Day 42 after transplantation was approximately 80% in all study strata including adults and children as well as children with malignant diseases, inborn errors of metabolism, and immunodeficiency syndromes. Factors adversely affecting engraftment or survival included lower cell doses, pretransplant cytomegalovirus seropositivity in the recipient, non-European ancestry, and greater HLA mismatching.

### Retrospective Studies

Over the nearly 15 years since the first unrelated-donor cord blood transplantation, more than 10,000 such procedures have been performed at more than 150 centers scattered throughout the world. Retrospective analyses of several of these patients have led to similar conclusions as the early studies. A minimum cell dose of  $3$  to  $3.5 \times 10^7$  nucleated cells/kg has been defined as a prerequisite for optimal clinical outcomes.<sup>13</sup> Efficacy of unrelated cord blood transplantation has been shown for children and adults with leukemias<sup>9,11</sup> and children with a variety of nonmalignant disorders including hemoglobinopathies,<sup>14</sup> immunodeficiencies,<sup>15,16</sup> and inborn errors of metabolism.<sup>17</sup> A majority of transplants have been mismatched at one or two HLA loci. Yet, a recent retrospective comparative analysis from the Center for International Blood and Marrow Transplant Research (CIBMTR) of unrelated donor graft sources—cord blood vs marrow—showed a comparable 5-year leukemia-free survival in children with acute leukemia who received allele-matched marrow vs one- or two-antigen mismatched cord blood transplants. The analysis suggests that 6/6 matched cord blood may yield superior survival, even though the comparison fell short of statistical significance.<sup>18</sup> Unfortunately, there have not been any prospective randomized clinical trials comparing unrelated cord blood and marrow to date.

### Inherited Metabolic Disorders and Unrelated UCB Transplantation

Cord blood transplantation has been particularly effective in the treatment of young infants and children with certain inborn errors of metabolism, eg, mucopolysaccharidoses such as Hurler syndrome and leukodystrophies such as Krabbe disease.<sup>17,19,20</sup> These diseases are examples of lysosomal storage

disorders, which result from single gene defects leading to specific enzyme deficiencies. These deficiencies cause defective lysosomal breakdown and accumulation of a toxic substrate, which in turn leads to progressive involvement of multiple organs, and eventually premature death. In these patients, durably engrafted cord blood cells of donor origin provide the missing or defective enzyme. Most patients with untreated Hurler syndrome usually die between 5 to 10 years of age from progressive cardiac and pulmonary involvement. They also suffer from severe bony abnormalities, corneal clouding, massive hepatosplenomegaly, and severe neuroregression. Engrafted cord blood cells have the ability to cross the blood-brain barrier and have been shown to effectively prevent neurodevelopmental progression, a beneficial outcome that is not expected with the alternative approach of recombinant enzyme replacement therapy.<sup>21</sup> Unrelated cord blood transplantation, when performed before the age of 2 to 3 years, leads to correction of cardiac, pulmonary, liver, and neurologic damage and improves survival.<sup>19</sup> In fact, a recent risk factor analysis of 146 patients from the European Group for Blood and Marrow Transplantation (EBMT) suggests that cord blood could be the preferred stem cell source in this disease.<sup>22</sup> Similarly, in patients with leukodystrophies, cord blood transplantation in the presymptomatic phase of the disease can prevent demyelination in the central and, often, the peripheral nervous systems.<sup>20</sup>

### Advantages and Disadvantages of Cord Blood Transplantation

Cord blood transplantation has several advantages over marrow or peripheral blood stem cell transplantation, the most significant of which is that cord blood transplantation can be performed successfully from unrelated donor with one or two HLA mismatches (Table 36-1). In the setting of marrow or

**Table 36-1.** Advantages and Disadvantages of Cord Blood

#### Advantages

- Increased immune tolerance
  - Less restrictive HLA barriers increase access to transplantation therapy for patients lacking fully matched donors
- Decreased incidence and severity of GVHD
- Increased safety
  - Less risk of viral contamination
  - Easier access
  - Ready availability
- Minimal donor attrition
- Easier recruitment of minority donors
- Noncontroversial source of stem cells

#### Barriers to Success

- Slower engraftment times
  - Possibly related to inadequate cell content in some single units, particularly for larger patients
- Cell dose limitations
- One-time usage
- Potential for transmission of genetic diseases
- Expensive infrastructure for banking



peripheral blood transplantation, however, this same degree of HLA mismatching causes unacceptable rates of acute and chronic GVHD and is prohibitive. With the current level of cord blood inventories and allowing for up to two antigen mismatches, a cord blood donor can be found for >90% of patients. There are other practical advantages to the use of allogeneic cord blood, such as increased certainty and promptness (<2 weeks vs 2 months) of obtaining the donor unit, less donor attrition, decreased risk of infections, absence of risk to mother or infant, and a higher likelihood of recruiting donors belonging to ethnic minorities. Last but not the least, cord blood may have more plasticity as suggested by the experience from transplantation in inherited metabolic disorders.<sup>19,22</sup>

There are also significant limitations dictated mainly by the kinetics of cord blood engraftment. The majority of single cord blood units may not have enough of a cell dose to be of benefit for adult patients. Delayed neutrophil and platelet engraftment often leads to more prolonged inpatient stay and higher acuity of supportive care, thus significantly increasing the cost of the transplant. Recent approaches combining two UCB units for a single transplant procedure have resulted in increased rates of engraftment in adults.<sup>23</sup> There is also a limited ability to perform posttransplant donor-derived cellular therapy such as donor lymphocyte infusions.

## Cord Blood Banking

The advances in clinical cord blood transplantation would not be possible without parallel evolution of a formal system of cord blood banking throughout the world. This involves collection of

the cord blood and subsequent processing and cryopreservation until it can be used clinically.

### Cord Blood Collection

Umbilical cord blood is the portion of the infant's blood contained in the placenta, which typically would be discarded at birth. As discussed, cord blood is a rich source of multipotent hematopoietic stem and progenitor cells. Properly performed, cord blood collection can be accomplished without physical risk to the mother or infant donor, from the delivered placenta (ex utero) or during the third stage of labor (in utero). Many public cord blood banks employ dedicated staff to perform ex-utero collections away from the delivery room so that the privacy of the family is preserved and clinicians are not distracted from their usual practices [Fig 36-1 (A)]. Alternatively, obstetricians or midwives perform in-utero collections while waiting for the placenta to deliver. In either case, after sterile preparation, the umbilical vein is punctured with a 17-gauge needle attached to a sterile, closed-system collection bag containing citrate-phosphate-dextrose anticoagulant which is positioned lower than the placenta [Fig 36-1 (B)]. Blood flows from the placenta, through the cord into the bag over approximately 5 to 10 minutes [Fig 36-1 (C)]. Experienced collectors harvest an average of 110 mL from a single placenta. The cord blood unit is labeled and subsequently shipped to the cord blood bank for processing, testing, cryopreservation, and storage.<sup>24</sup>

There are two types of cord blood banks—public and private. Unrelated transplant programs employ public banks as their source of donor UCB units. These units are donated on a voluntary basis by women delivering healthy infants at term. Private banks, which are for-profit entities, store “directed donations”

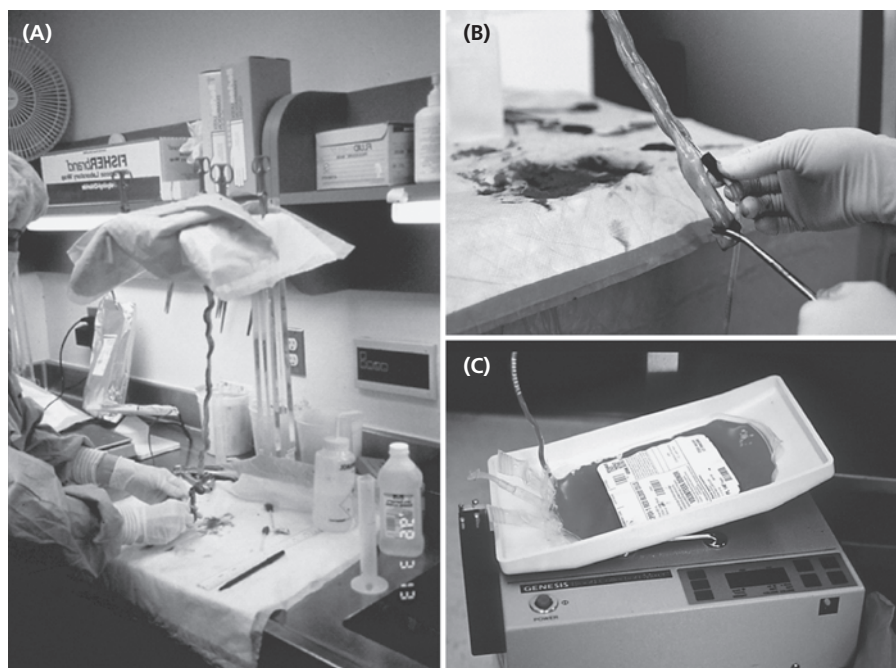


**Figure 36-1.** Ex-utero cord blood collection. The placenta is delivered by usual obstetric techniques. After cutting and clamping the cord, operating room personnel place the placenta in a bucket and transport it to the collection room. There it is placed in a collection stand (A) on a clean chuck pad. The fetal side is placed down and the umbilical cord is pulled through an opening in the tray. The cord is tethered, cleansed with betadine and alcohol, and punctured with a 17-gauge needle attached to a collection bag (B). Blood drains by gravity over 5 to 10 minutes into the attached bag, which contains 25 mL of citrate-phosphate-dextrose anticoagulant (C). After the blood collection is complete, the needle is removed from the cord, the tubing is sealed, and the cord blood unit is transported to the processing laboratory for volume reduction, testing, cryopreservation, and long-term storage.

collected by obstetricians from infants born into families who intend to use the cord blood for the infant from whom it came (autologous donation) or for another family member in need of future transplantation therapy.

Cord blood collection in public cord blood banks today requires obtaining maternal consent. Guidelines for several aspects of cord blood banking are provided by the Institute of Medicine (IOM).<sup>25</sup> By consenting, the infant's mother agrees to the following:

1. The donation of her infant's cord blood is voluntary.
2. She gives permission for her blood and the cord blood to be tested for blood-borne pathogens, eg, human immunodeficiency virus, hepatitis B and C viruses, syphilis, human T-cell lymphotropic virus, and West Nile virus, and agrees to give a detailed family medical history.
3. The cord blood is not being stored for personal use by the infant or other relatives and, instead, it will be listed on a public registry of unrelated donors and made available to patients in need of donors for unrelated transplantation.
4. She may be contacted in the future by the bank to obtain follow-up information on the infant's health.
5. Measures will be used to protect her confidentiality and that of her infant.

Unlike adult donor registries, the identities of cord blood donors are not revealed to the recipients and a recipient cannot contact the donor in the future. Most banks maintain a confidential link between the unit information (stored as a bar-code label) and the mother's demographic information with the aim of being able to contact the family in the future, should the unit test positive for a new disease not anticipated by the family.

Timing of maternal consent can vary. The vast majority of cord blood banks believe that consent should be obtained from the mother before collection of the cord blood, preferably in the late third trimester and before onset of labor. Despite efforts to recruit women during the third trimester of pregnancy and obtain their consent well before the onset of labor, many pregnant women in labor come to the hospital interested in cord blood donation but without having given prior consent. Some centers have addressed this situation by asking pregnant women in early labor to sign a short or "mini" consent form allowing only the collection of the cord blood and maternal samples. Then after she recovers from the delivery someone meets with her to educate her and obtain full consent allowing for processing, testing, cryopreservation, and storage.

### Cord Blood Storage

As commercial, private cord blood banking has proliferated, its ethical justification has been widely debated.<sup>26,27</sup> Generally, an initial storage fee of \$1000-1500 is charged, followed by a yearly storage fee of approximately \$100. While there are a few clear indications for this practice (eg, a sibling with cancer, a family history of hemoglobinopathy, marrow failure, congenital immunodeficiency syndrome, or inborn error of metabolism), the vast majority of families who store cord blood with private banks

pay to have access to stem cells in the future for use in treating degenerative diseases or problems related to injuries or aging. Currently, there is no evidence that this future use will be feasible or efficacious in such circumstances.

Many private banks aggressively advertise their services. References in such advertising to rare and not-yet-tested applications for cord blood transplantation address the concerns of new parents wanting to provide every possible advantage for their newborn child.<sup>28</sup> In addition to messages that may be exploitative, marketing may be inaccurate or misleading. One common reason offered by some private banks for storing autologous cord blood is to have a source of stem cells for transplantation if the child were to develop leukemia. However, most children with childhood leukemias can be cured with conventional chemotherapy alone and in those who do not respond to this approach, allogeneic transplantation is the treatment of choice. Furthermore, leukemic cells have been found in autologous cord blood of infants who later present with leukemia from 1 to 11 years of age.

With increasing use of cord blood transplantation, public banks face other challenges. Procuring and providing units for public use in unrelated allogeneic transplantation has involved funding from third party sources for the creation of the inventory. Currently, there are approximately 14 public cord blood banks in the United States and approximately 30 more worldwide. All of these banks struggle financially because the revenues gained from sale of UCB units for transplantation are not sufficient to support the basic operations of a bank that is in the process of building inventory. There is also no requirement for public cord blood banks to list their inventories on a single registry available to all transplant centers. Thus, transplant centers must be knowledgeable about and search multiple banks and registries to find the best donor for each patient.

In 2004, after appropriation of \$20,000,000 by the US Congress to increase the inventory of cord blood units in US public banks, the Health Resources Services Administration (HRSA) asked the IOM to perform a study to determine the best way to organize public cord blood banking and distribution to patients undergoing unrelated transplantation, the results of which were published in April 2005.<sup>25</sup> In brief, the IOM recommended that HRSA contract with eligible banks to procure approximately 150,000 new, ethnically diverse, unrelated donor cord blood units over the next 5 years. The units will need to meet quality standards as defined by an advisory board, the Food and Drug Administration, and other accrediting agencies. They will also have to be listed on a computerized, Web-based system created to allow searching of all unrelated cord blood and adult donors from a single point of access (SPA).

Since the publication of the IOM study, the US Congress passed legislation that appropriated funds to establish a National Cord Blood Program. The WC "Bill" Young Stem Cell Transplantation Act, is federally funded and administered through HRSA. Selected cord blood banks have been chosen to participate in the National Cord Blood Inventory, which is

tasked with building a high-quality, ethnically diverse inventory of 150,000 new UCB units to be listed on a combined registry for adult and cord blood donors. Under this new program, patient advocacy must be provided and transplant outcomes collected and evaluated. There is also provision for a SPA for patients and doctors searching for a suitable stem cell HLA match—be it from marrow, peripheral blood, or cord blood. The legislation also instituted mandatory reporting of transplant outcomes by all transplant centers in the United States to the SCTOD (Stem Cell Transplant Outcomes Database), a contract awarded to the CIBMTR. HRSA administers contracts for the banks directly, whereas the other programs (adult stem cell and cord blood coordinating centers, SPA, and SCTOD) were awarded to, and are all administered through, the NMDP.

## Future Directions

Umbilical cord blood from related and unrelated donors, matched or mismatched at one or two antigens, is now widely regarded as an alternate donor source to matched marrow or peripheral blood for allogeneic transplantation in children as well as adults for a variety of malignant and nonmalignant disorders. This use of cord blood dramatically increases the accessibility of hematopoietic stem cell transplantation. Retrospective analysis of large patient datasets does not show a survival difference and prospective randomized clinical trials comparing these graft sources are needed. Despite its favorable impact on the field of hematopoietic transplantation, time to engraftment remains a weakness and strategies to improve engraftment rates and speed of engraftment are needed. Several new approaches are being investigated, including combining two cord blood units<sup>23</sup> and combining a cord blood unit with cells from a haploidentical donor.<sup>29</sup> Investigations are also ongoing to study reduced-intensity conditioning regimens in order to decrease the toxicity associated with transplantation. A federal mandate has provided an impetus for the cord blood banking community to increase the cord blood inventories throughout the country and to streamline efforts into creation of a national cord blood banking program. Increasing inventories, especially for minorities, will result in availability of better matched cord blood units and improved outcomes in this segment of the population. These developments in cord blood banking are thus expected to complement the growth of clinical allogeneic cord blood transplantation. There has been increasing new evidence in recent years suggesting presence of pluripotent cells in cord blood that hold vast potential in treatment of degenerative disorders involving several organs other than hematopoietic system, such as liver, brain, heart, eyes, endocrine organs, and bones.<sup>30</sup>

## Disclaimer

The authors have disclosed no conflicts of interest.

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## 37

# Alternatives to Transfusion: Perioperative Blood Management

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Recognition of blood supply limitations and concern for potential deleterious effects of transfusion have promoted the concept of integrated programs for blood management in the surgical setting.<sup>1</sup> Perioperative transfusions account for more than half of the estimated 20 million blood components transfused annually.<sup>2</sup> Logically, if measures are taken to reduce this utilization, both the pressure on resource allocation as well as the risks associated with allogeneic transfusion could be reduced. The scope of blood management is broad and includes several different options, some of which can be initiated before patient hospitalization, others of which occur during the intraoperative period or the postoperative period (Table 37-1). This chapter covers the various aspects of blood management, which present alternatives to allogeneic transfusion.

**Table 37-1.** General Principles of Blood Management

1. Formulate a plan of care for avoiding and controlling blood loss tailored to the clinical management of individual patients, including anticipated and potential procedures.
2. Employ a multidisciplinary treatment approach to blood conservation using a combination of interventions.
3. Manage blood utilization proactively and be prepared to address potential complications.
4. Promptly investigate and treat anemia, preferably preoperatively.
5. Exercise clinical judgment and be prepared to modify routine practice when appropriate.
6. Consult promptly with senior specialists experienced in blood conservation at an early stage if there is physiologic deterioration or if complications arise.
7. Restrict blood drawing for laboratory tests.
8. Decrease or avoid the perioperative use of anticoagulants and antiplatelet agents.

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*Rossi's Principles of Transfusion Medicine, 4th Edition*, Edited by Toby L. Simon, E. L. Snyder, B. G. Solheim, Christopher, P. Stowell, Ronald, G. Strauss and Marian Petrides © 2009 Blackwell Publishing Ltd. ISBN: 978-1-405-17588-3

## Blood Management in the Preoperative Period

Blood management strategies should begin during the preoperative phase of patient management. During the preoperative evaluation of a patient, it is important to carefully review a patient's personal and family history to assess for potential bleeding risks. For example, family history of von Willebrand disease or specific factor deficiency (eg, Factor XI deficiency) identifies a patient who is potentially at risk for significant blood loss. Also important is the review of medication history for presence of drugs that interfere with either primary or secondary hemostasis (eg, aspirin, aspirin-containing compounds, antiplatelet agents, anticoagulants). Additionally, evidence of anemia, either through history or laboratory assessment, should be determined. If present, measures to increase red cell mass before surgery should be recommended (eg, nutritional supplementation with iron).<sup>1</sup>

## Autologous Blood Donation

The practice of preoperative autologous blood donation (PAD) gained widespread use in the early 1980s when there was a growing concern about transfusion-transmitted hepatitis and human immunodeficiency virus.<sup>3-5</sup> However, several reviews of PAD programs suggest that their role in transfusion management has declined, an observation confirmed by national data on blood procurement (Table 37-2).<sup>6-9</sup> The decline is attributed to reduced risk for transfusion-transmitted disease and improved surgical techniques leading to reduced blood loss, as well as the acceptance of lower transfusion thresholds.<sup>6</sup> Further questions about the efficacy of PAD were raised in the systematic review published by the Cochrane Collaboration.<sup>10</sup> Results of this review showed that although PAD is associated with an overall reduction of allogeneic blood exposure, patients participating in PAD have a higher likelihood of transfusion (allogeneic and autologous blood) compared with patients who do not participate in PAD. Reasons for this include lower preoperative hemoglobin concentrations, resulting in increased probability

**Table 37-2.** Collection and Transfusion of Autologous Blood (in Thousands of Units) in the United States between 1999 and 2006<sup>9</sup>

|                   | 1999   | 2001   | Percent<br>Change<br>1999-2001 | 2004   | Percent<br>Change<br>2001-2004 | 2006   | Percent<br>Change<br>2004-2006 |
|-------------------|--------|--------|--------------------------------|--------|--------------------------------|--------|--------------------------------|
| <b>Collected</b>  |        |        |                                |        |                                |        |                                |
| Autologous        | 659    | 619    | −4.8                           | 458    | −26.0                          | 335    | −26.9                          |
| % of total        | 4.8    | 4.1    |                                | 3.0    |                                | 2.1    |                                |
| Total             | 13,649 | 15,076 |                                | 15,019 |                                | 16,023 |                                |
| <b>Transfused</b> |        |        |                                |        |                                |        |                                |
| Autologous        | 367    | 359    | −2.3                           | 270    | −24.6                          | 189    | −30.0                          |
| % of total        | 3.0    | 2.6    |                                | 1.9    |                                | 1.3    |                                |
| Total             | 12,389 | 13,898 |                                | 14,182 |                                | 14,650 |                                |

of requiring intraoperative and/or postoperative blood transfusion and, possibly, more liberal use of transfusion based on the fact that there is “blood in the bank.”<sup>11</sup> The findings of Billote et al,<sup>12</sup> in particular, suggest that PAD has limited utility for reducing the need for allogeneic transfusion, even for procedures associated with significant blood loss. In this well-designed, randomized, controlled study the investigators examined the usefulness of PAD for reducing the need for allogeneic transfusion in patients undergoing unilateral primary total hip replacement. The patient population consisted of surgical candidates who were not anemic (predonation hemoglobin greater than or equal to 12 g/dL) and the collection protocol required that the last unit be collected no later than 2 weeks before surgery. Additionally, patients with significant comorbidities (including severe or unstable cardiac disease, uncontrolled hypertension, symptomatic carotid or vertebral artery stenosis, a history of bleeding diathesis, or bacteremia) were excluded from the study. Standardized transfusion guidelines for allogeneic and autologous transfusion used intraoperatively, included: 1) acute blood loss (>25% blood volume), 2) tachycardia attributed to hemoglobin-responsive hypoxia and unresponsive to intravenous fluid administration, 3) a hemoglobin concentration <7 g/dL in healthy patients, and 4) a hemoglobin concentration of <8 g/dL in patients with significant comorbid disease (eg, heart disease). In the postoperative period, indications for autologous transfusion included a hemoglobin concentration of <11 g/dL in the immediate postoperative recovery period and <10 g/dL in the subsequent postoperative period. As summarized in Table 37-3, important findings of this study included the observation that no patient required allogeneic transfusion; furthermore, patients randomly assigned to the nondonation cohort did not require transfusion, whereas those in the PAD cohort received a total of 42 autologous Red Blood Cell (RBC) units. Also significant was that 34 (41%) of the 82 autologous units collected were not transfused.<sup>13</sup> This report has led many in the field of transfusion medicine to question the role of PAD in blood conservation, particularly because it is associated with a relatively high wastage of units.

**Table 37-3.** Randomized Controlled Trials Examining Autologous vs Allogeneic RBC Use in Patients Undergoing Hip Replacement Surgery

| Variable                              | Cohort     |            |
|---------------------------------------|------------|------------|
|                                       | Autologous | Allogeneic |
| Number of patients                    | 42         | 54         |
| Number of autologous units transfused |            |            |
| 0                                     | 13         | 0          |
| 1                                     | 10*        | 0          |
| 2                                     | 19*        | 0          |
| Allogeneic units transfused           | 0          | 0          |

\*34 (41%) of the 82 available autologous units were not transfused.

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### Considerations for a Successful PAD Program

Traditionally, PAD has been advocated for patients who are undergoing procedures associated with significant blood loss (procedures associated with >90% likelihood of requiring transfusion), such as orthopedic joint replacement, cardiothoracic procedures, vascular surgery, and radical prostatectomies.<sup>14</sup> In contrast, PAD is unnecessary for patients scheduled for procedures with little anticipated blood loss, for example, vaginal hysterectomies.<sup>14</sup> To optimize the success of PAD, several factors should be considered. Studies have shown the endogenous erythropoietic response to PAD is modest, and can be negatively affected by poor nutritional status and/or anemia of chronic disease. Thus, an assessment for history of chronic disease or chronic blood loss, as well as general nutritional status should be performed to determine if PAD is appropriate. Based on several studies (Table 37-4), the standard phlebotomy schedule of 1 unit/week in patients who are otherwise healthy (nonanemic and iron replete) can lead to an 11% to 19% expansion of red cell mass (equivalent to 1.0–1.75 RBC units).<sup>16,17</sup> More aggressive phlebotomy programs (eg, 2 units/week) promote a more robust erythropoietic response (equating to 2–3 RBC units) that is enhanced with the addition of exogenous erythropoietin administration.<sup>18–21</sup>

Table 37-4. Erythropoiesis during Autologous Blood Donation

| Patients (n)          | Baseline Red Cell Mass (mL) | Blood Removed (Donated) |         |          | Blood Produced   |            | Iron Therapy | Ref                              |
|-----------------------|-----------------------------|-------------------------|---------|----------|------------------|------------|--------------|----------------------------------|
|                       |                             | Units                   |         | RBC (mL) | Red Cell Mass mL | Increase % |              |                                  |
|                       |                             | Requested               | Donated |          |                  |            |              |                                  |
| Standard phlebotomy   |                             |                         |         |          |                  |            |              |                                  |
| 108                   | 1,884                       | 3                       | 2.7     | 522      | 351              | 19%        | PO           | Kasper et al <sup>16</sup>       |
| 22                    | 1,936                       | 3                       | 2.8     | 590      | 220              | 11%        | None         | Kasper et al <sup>17</sup>       |
| 45                    | 1,881                       | 3                       | 2.9     | 621      | 331              | 17%        | PO           | Kasper et al <sup>17</sup>       |
| 41                    | 1,918                       | 3                       | 2.9     | 603      | 315              | 16%        | PO, IV       | Kasper et al <sup>17</sup>       |
| Aggressive phlebotomy |                             |                         |         |          |                  |            |              |                                  |
| 30                    | 2,075                       | ≥3                      | 3.0     | 540      | 397              | 19%        | None         | Weisbach et al <sup>18</sup>     |
| 30                    | 2,024                       | ≥3                      | 3.1     | 558      | 473              | 23%        | PO           | Weisbach et al <sup>18</sup>     |
| 30                    | 2,057                       | ≥                       | 2.9     | 522      | 436              | 21%        | IV           | Weisbach et al <sup>18</sup>     |
| 24                    | 2,157                       | 6                       | 4.1     | 683      | 568              | 26%        | PO           | Goodnough et al <sup>19,20</sup> |
| 23                    | 2,257                       | 6                       | 4.6     | 757      | 440              | 19%        | PO           | Goodnough et al <sup>21</sup>    |

Data expressed as means.  
PO = oral; IV = intravenous  
\* Reprinted with permission from Goodnough et al.<sup>15</sup>

Complications and Adverse Effects of PAD

The incidence of donor reactions in the autologous blood donor population appears to be similar to that of allogeneic donors, ranging from 2% to 5%.<sup>22,23</sup> As with allogeneic donations, risk factors include first-time donation, female gender, and decreasing age.<sup>23</sup> The majority of reactions are mild and generally vasovagal in origin<sup>11</sup>; however, recent assessments question whether the side effects merit the time and effort put into autologous donation, particularly in the pediatric population.<sup>24</sup> A recent study demonstrated a dramatic reduction (21%) in donor reactions by the simple ingestion of 0.5 L of water within 10 minutes of the donation episode.<sup>25</sup>

The transfusion of autologous blood has many of the same complications as transfusion of allogeneic units, including bacterial contamination, hemolysis resulting from errors in the administration of units, and volume overload. Advantages and disadvantages of PAD are summarized in Table 37-5. Because mortality from allogeneic blood transfusion is now more likely the result of administrative error<sup>26</sup> rather than blood-transmitted infection,<sup>27</sup> the risks of banked autologous blood units are similar to banked allogeneic blood units.

Erythropoietin

Erythropoietin, as discussed in Chapter 2, is a key regulator of erythropoiesis. Briefly, endogenous erythropoietin is released by renal cortical interstitial cells in response to tissue hypoxia.<sup>28</sup> The released hormone then exerts an effect on colony-forming unit erythroid precursors in the marrow, promoting cellular differentiation and maturation.<sup>28</sup> Additional actions include enhanced hemoglobin synthesis and release of reticulocytes into the systemic circulation.<sup>28</sup>

Table 37-5. Autologous Donation

| Advantages                                                    | Disadvantages                                                               |
|---------------------------------------------------------------|-----------------------------------------------------------------------------|
| 1. Prevents transfusion-transmitted disease                   | 1. Risk of bacterial contamination or volume overload remains               |
| 2. Prevents red cell alloimmunization                         | 2. Does not eliminate risk of administrative error with ABO incompatibility |
| 3. Supplements the blood supply                               | 3. More costly than allogeneic blood                                        |
| 4. Provides compatible blood for patients with alloantibodies | 4. Wastage of blood not transfused                                          |
| 5. Prevents some adverse transfusion reactions                | 5. Causes perioperative anemia and increased likelihood of transfusion      |

As noted above, the erythropoietic response to blood loss anemia is modest. The discovery that compensatory hematopoiesis is better enhanced through the administration of exogenous erythropoietin (recombinant erythropoietin) spawned several clinical trials investigating the role of erythropoietin therapy in the preoperative setting. Patients scheduled to undergo surgical procedures associated with significant blood loss (eg, major orthopedic, cardiovascular, and urologic surgical procedures) were specifically evaluated.<sup>29-35</sup> As summarized in Table 37-6, the clinical trials in the orthopedic patient population, using a variety of dosing regimens, demonstrated benefit; preoperative hemoglobin concentrations increased and allogeneic transfusions were reduced. Patients with preoperative hemoglobin concentrations ranging between 10 and 13 g/dL appeared to benefit the most from preoperative

**Table 37-6.** Efficacy of Perioperative Recombinant Erythropoietin in Major Elective Orthopedic Surgery

| Study (Number of Patients)           | Orthopedic Procedure      | EPO Dose                                                                                              | Oral Iron                               | Key Findings                                                                                                                                                           |
|--------------------------------------|---------------------------|-------------------------------------------------------------------------------------------------------|-----------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Canadian Study <sup>29</sup> (198)   | Hip arthroplasty          | 300 IU/kg $\times$ 14 or 9 days vs placebo                                                            | 300 mg beginning 21 days before surgery | Dose-dependent increase in hemoglobin concentration and reticulocyte count<br>Decrease in risk of exposure to allogeneic blood in patients treated with erythropoietin |
| Faris et al <sup>30</sup> (185)      | Hip and knee arthroplasty | 300 IU/kg or 100 IU/kg $\times$ 15 days vs placebo                                                    | 325 mg TID while on study               | Dose-dependent increase in hemoglobin concentration and reticulocyte count<br>Decrease in risk of exposure to allogeneic blood in patients treated with erythropoietin |
| de Andrade et al <sup>31</sup> (290) | Hip and knee arthroplasty | 300 IU/kg or 100 IU $\times$ 15 days vs placebo                                                       | Yes                                     | Dose-dependent increase in hemoglobin concentration and reticulocyte count<br>Decrease in risk of exposure to allogeneic blood in patients treated with erythropoietin |
| Goldberg et al <sup>32</sup> (140)   | Hip and knee arthroplasty | 600 IU/kg, weekly for 3 weeks before surgery and once on day of surgery<br>300 IU/kg $\times$ 15 days | Yes                                     | Increase in hemoglobin concentration<br>Equivalent response with weekly vs daily dosing regimen in reducing allogeneic transfusion                                     |

\* Modified with permission from Faris and Ritter.<sup>28</sup>

administration of erythropoietin.<sup>31</sup> On the basis of the results of these clinical trials, erythropoietin therapy gained US regulatory approval in 1996 for perioperative use in patients scheduled for elective, noncardiovascular surgical procedures. Several trials in orthopedic patient populations have confirmed the positive impact of erythropoietin administration on reducing allogeneic red cell transfusion, particularly when coupled with iron repletion.<sup>36,37</sup> However, despite the evidence of benefit, the use of erythropoietin has not been widely adopted. Deterrents to the general use of erythropoietin include the inconvenience of self-administration, restrictive reimbursement by insurance companies, concern for thrombosis (particularly in the cardiovascular surgical patient population<sup>38</sup>), and lack of demonstrable cost-effectiveness with respect to patient morbidity and mortality.<sup>39</sup> However, as the enthusiasm for PAD declines, it is possible that more general use of erythropoietin in the preoperative setting will increase.

## Intraoperative/Postoperative Strategies

### Acute Normovolemic Hemodilution

Acute normovolemic hemodilution (ANH) is the immediate preoperative removal of whole blood and its replacement with an

acellular fluid (in general, a combination of crystalloid and colloid) in order to maintain a normovolemic state. The practice stemmed from the early days of open-heart surgery when “blood pooling,” the intentional removal of whole blood, was employed as a means to sequester platelets, thus avoiding contact activation via exposure to the extracorporeal circuit and consequent development of postoperative coagulopathies.<sup>40,41</sup> Soon thereafter, it was recognized that additional potential benefits of ANH included a reduction of red cell loss, because the hematocrit of the blood shed during intraoperative procedures is less. For example, in theory, a 2-L blood loss in a patient with a 45% hematocrit, or the equivalent of 900 mL of red cells, could be reduced to 400 mL of red cells following hemodilution to a hematocrit of 20%. Several reports illustrating the mathematical modeling of hemodilution suggest that the benefit of ANH, in terms of reduction in significant red cell loss, is realized only in cases where extreme hemodilution (eg, a reduction in hematocrit to 21% or lower) is undertaken and the surgical blood loss volume is large (in excess of 1.5 L) (Fig 37-1).<sup>43-45</sup>

### Safety and Efficacy of ANH: Clinical Studies

ANH is quite a straightforward procedure. Following induction of anesthesia, blood is withdrawn into standard blood collection bags containing anticoagulant-preserved solution. Upon



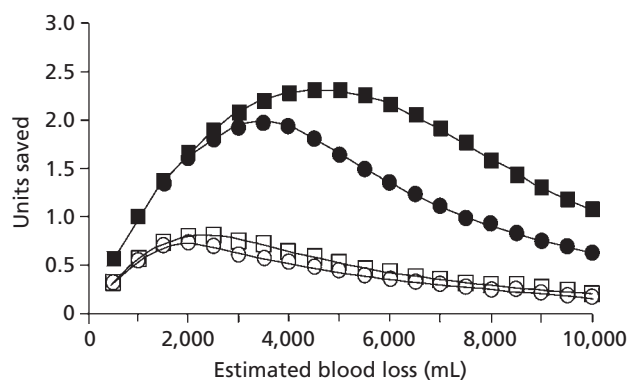


**Figure 37-1.** The theoretic relationship between the number of Red Blood Cell units saved as a function of estimated blood loss following acute normovolemic hemodilution to a minimum hematocrit of 28% (open symbols) or a hematocrit of 18% (shaded symbols) for a patient with a blood volume of 5 liters and an initial hematocrit of 45%. Replacement strategies were either to infuse mL-for-mL of blood lost (circle symbols) or to transfuse so as to maintain the hematocrit at the minimal hematocrit (square symbols).

(Reprinted with permission from Goodnough et al.<sup>42</sup>)

completion of the blood collection process, the units are labeled with the patient's name and stored at room temperature within the operative suite. The collected units of blood are then reinfused to the patient at the close of the procedure, in reverse order. The first unit collected, and therefore the last unit transfused, has the highest hematocrit and concentration of clotting factors and platelets. Although ANH decreases the arterial oxygen content of the circulating blood, compensatory mechanisms including increased cardiac output and decreased systemic vascular resistance might be expected, in theory at least, to maintain adequate tissue oxygen delivery (Table 37-7).<sup>46</sup>

Mathematical models suggest that ANH is not very effective in reducing the amount of red cells lost, and may put some patients at risk for untoward complications related to iatrogenically induced ischemia.<sup>43</sup> Clinical studies, as well, have shown little sparing of allogeneic RBC transfusion with the use of ANH. In a recent meta-analysis, Siegel et al<sup>47</sup> examined 42 studies of ANH that met the inclusion criteria of being randomized, controlled trials of either large-volume or low-volume ANH in a surgical population. The studies were conducted from 1972 through 2002, and a majority involved patients undergoing cardiothoracic surgery, followed by trials in hip arthroplasty. It was evident from this analysis that there was an insignificant reduction in the relative risk (RR) for allogeneic transfusion for patients managed with ANH [RR, 0.96; 95% confidence interval (CI), 0.90-1.01]. A study of selected patients undergoing hip arthroplasty demonstrated no significant reduction in the number of allogeneic transfusions in patients randomly assigned to ANH vs standard transfusion therapy.<sup>48</sup> The authors appropriately point out that their data may have been affected by transfusion practices that were more conservative than expected (ie, acceptance of a lower hemoglobin concentration), inability to accomplish ANH in all patients randomly assigned to the ANH arm because of technical

**Table 37-7.** Compensatory Mechanisms Following Hemodilution and the Creation of an Acute Anemia

- Increased cardiac output
- Increased stroke volume
- Slight increase in heart rate (not clinically significant)
- Reduced blood viscosity and systemic vascular resistance leading to decreased afterload
- Increase blood flow to oxygen-dependent tissues—beyond the cardiac output increase
- Increase in oxygen extraction in end organs
- Increased coronary blood flow

Used with permission from Shander and Rijhwani.<sup>46</sup>

difficulties (primarily poor venous access precluding blood collection), and protocol violations (particularly in the ANH arm). The study of Sanders et al<sup>49</sup> in which patients scheduled to undergo major gastrointestinal surgery for which total blood losses were anticipated to be >40%, also failed to demonstrate benefit of ANH vs standard transfusion practice with respect to reduced allogeneic blood transfusion in the operative or postoperative period. Still other studies have supported the use of ANH as a means to avoid allogeneic transfusion. Casati et al<sup>50</sup> reported on a study of patients undergoing off-pump coronary artery bypass surgery who were randomly assigned to undergo either ANH or standard transfusion management. The protocol included ANH of 17% total blood volume (moderate ANH), use of red cell recovery and infusion, and a restrictive transfusion strategy (hemoglobin of 8 g/dL). Patients undergoing ANH received statistically significantly fewer RBC units and were more likely to have been transfused as a consequence of surgical bleeding rather than postoperative anemia. The authors concluded that ANH offers the potential to reduce allogeneic red cell exposure in this particular surgical population. The mixed reports regarding the clinical effectiveness of ANH as a conservation strategy result, in large part, from the heterogeneity in the methods of ANH (large volume vs moderate volume), the surgical procedures, and the transfusion management protocols. This inconsistency signals the need for larger and more rigorous clinical trials examining the benefit of ANH.<sup>51</sup>

Although efficacy of ANH is not clearly established, it poses little risk of harm to the patient. Furthermore, it appears to be a low-cost procedure compared to other alternatives directed at procurement of autologous blood.<sup>51</sup>

### Intraoperative Autologous Blood Recovery and Reinfusion

Intraoperative red cell recovery and reinfusion of autologous blood represent another approach to reduction in allogeneic transfusion. In general, their use has been restricted to surgical procedures in which 1) the estimated blood loss is anticipated to be more than 20% of a patient's blood volume or 2) greater than 10% of patients undergoing the procedure require transfusion

**Table 37-8.** General Indications for Use of Intraoperative Blood Recovery Procedures\*

| Specialty    | Surgical Procedure                      | Comments                                                        |
|--------------|-----------------------------------------|-----------------------------------------------------------------|
| Cardiac      | Valve replacement                       |                                                                 |
|              | Redo bypass grafting                    |                                                                 |
| Orthopedics  | Major spine                             |                                                                 |
|              | Bilateral knee replacement              |                                                                 |
|              | Revision hip replacement                |                                                                 |
| Urology      | Radical retropubic prostatectomy        | Individualized by surgeon                                       |
|              | Cystectomy                              | Limited to patients with prior radiation therapy                |
|              | Nephrectomy                             | When tumor involved major vessels                               |
|              |                                         |                                                                 |
| Neurosurgery | Giant aneurysm                          |                                                                 |
| Vascular     | Thoracoabdominal aortic aneurysm repair |                                                                 |
|              | Abdominal aortic aneurysm repair        | Should be individualized by surgeon and patient characteristics |
| Other        | Jehovah's Witness                       | When accepted by patient                                        |
|              | Unexpected massive blood loss           |                                                                 |
|              | Red cell antibodies                     |                                                                 |
|              |                                         |                                                                 |

\*Reprinted with permission from Waters.<sup>52</sup>

(and the mean transfusion requirement is >1 unit). Table 37-8 lists commonly accepted indications. Recovery techniques include collection of blood by direct suctioning from the operative field as well as recovery of blood from surgical sponges. Following recovery, the blood may or may not be further manipulated. Devices available for postcollection processing of shed blood fall into two main categories: 1) hemofiltration, whereby shed blood is drawn into the autotransfusion device, which is equipped with an integral filtration device; the collected product is then simply filtered (ie, there is no washing of the product) and reinfused to the patient<sup>52</sup> and 2) red cell wash systems, whereby shed blood is collected and subsequently processed by automated devices that wash the shed blood free of cellular debris and other contaminants as well as concentrate the recovered red cells.<sup>52,53</sup>

### Efficacy

The evidence that use of cell recovery and reinfusion promotes avoidance of allogeneic blood transfusion is not solid. A recent review of 51 published trials on cell recovery and reinfusion involving primarily cardiac and orthopedic patients revealed several methodologic problems in this area of investigation, including small numbers of study subjects, heterogeneity in transfusion practice (protocol-driven vs transfusion at the investigators' discretion), and either no reference to whether the study was blinded, or clear indications that the study was not blinded.<sup>54</sup> Despite these concerns, the authors, after applying rigorous review criteria,

**Table 37-9.** Possible Contraindications to Intraoperative Blood Recovery\*

### Pharmacologic agents

Clotting agents (Avitene, Surgicel, Gelfoam, etc)  
Irrigating solutions (Betadine, antibiotics meant for topical use)  
Methylmethacrylate  
Anticoagulants

### Contaminants

Urine  
Bone chips  
Fat  
Bowel contents  
Infection  
Amniotic fluid  
Cellular stroma  
Activated leukocytes, platelets, complement, plasmin

### Malignancy

### Hematologic disorders

Sickle cell disease  
Thalassemia

### Miscellaneous

Carbon monoxide (electrocautery smoke)  
Catecholamines (pheochromocytoma)  
Oxymetazoline (Afrin)

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determined that use of red cell recovery and reinfusion reduced the relative risk for perioperative allogeneic RBC transfusion exposure by 39% (RR = 0.61; 95% CI, 0.52 to 0.71). Furthermore, their analysis showed a more pronounced reduction for allogeneic red cell exposure in orthopedic patients vs cardiac patients (58% compared to 23%, respectively). A critical question is whether a reduction in the relative risk for allogeneic red cell exposure equates to a meaningful reduction in volume of allogeneic red cell transfusion. Unfortunately, of the 51 studies analyzed, only 27 reported data on the volume of allogeneic red cells transfused. Based on these available data, use of cell recovery and reinfusion reduced the average number of allogeneic RBC transfusions by only 0.67 unit/patient (95% CI, 0.89 to 0.45 unit). However, when analyzed in terms of a carefully constructed economic model, the cost/benefit ratio of intraoperative recovery supported its use as an effective measure to reduce allogeneic red cell exposure.<sup>55</sup>

### Complications

Although seemingly straightforward, transfusion of recovered blood is not without potential risks, and for some clinical situations, the practice is contraindicated (Table 37-9). However, it is important to recognize that many contraindications are relative, not absolute, because there are limited data to support the danger of cell recovery in these situations.<sup>52</sup> For example, studies of tumor surgeries in which cell recovery has been employed as a blood conservation measure have not demonstrated an increase

in either incidence of metastatic disease or death, but have resulted in reduced allogeneic red cell transfusion.<sup>57-59</sup>

The literature is peppered by reports of disseminated intravascular coagulation and acute renal failure secondary to reinfusion of recovered blood that was either filtered or inadequately washed, improper use of the devices, inadvertent use of hypo- or hypertonic wash solutions resulting in compromised products, and air embolism caused by inadequate venting of collection/infusion bags.<sup>52</sup> Patient factors appear to play a major role in risk for complications; for example, concern for bacterial contamination of recovered blood is probably valid in a case of major abdominal trauma with significant bowel injury, but not as much in elective cases of abdominal surgery, where the surgical setting is much more controlled.<sup>60</sup> Similarly, the concern for renal damage secondary to hemolyzed red cells following recovery may be warranted in patients with underlying renal pathology or in patients who are hypotensive, thus at risk for suboptimal renal perfusion.<sup>60</sup> Such concerns may be more valid for postoperatively recovered blood, where hemofiltration devices are more commonly used.<sup>61</sup>

### Postoperative Autologous Blood Recovery and Reinfusion

Postoperative recovery of blood involves the collection of blood from surgical drains followed by reinfusion, with or without processing. Blood recovered by hemofiltration (in contrast to systems where recovered products are washed as part of the recovery process) can be dilute, partially hemolyzed, and contain high concentrations of cytokines and cellular debris.<sup>62-64</sup>

The safety and the benefit of the use of unwashed blood obtained from surgical drains following orthopedic surgery remain in question.<sup>65</sup> Although the results of a recent randomized clinical trial<sup>66</sup> evaluating the effectiveness and safety of postoperative recovered blood demonstrated a reduction in allogeneic transfusion in the study arm (vs control), this study was plagued by several confounding variables, including mixed surgical types with unequal randomization, lack of characterization of the recovered product, and variable transfusion thresholds.<sup>65</sup> Similar concerns about heterogeneity of study population and variable transfusion practices were also highlighted by Carless et al<sup>54</sup> in their systematic review of perioperative cell recovery and suggest the need for further study of the role of such devices in perioperative blood management.

### Summary

The blood needs of surgical patients can be managed by many alternatives to allogeneic transfusion. However, several factors (eg, patient baseline hematocrit, cardiac performance, anticipated surgical blood loss) affect their practicality of use and cost-effectiveness. Hence, effective use of these alternatives, either alone or in combination, requires the prospective identification of appropriate surgical candidates who will need transfusion and

who may truly benefit from blood conservation. The primary impetus to avoid allogeneic transfusion is no longer based on the safety of the blood supply, but rather on evidence that blood conservation is safe and of value for individual patients.

### Disclaimer

The author has disclosed a financial relationship with Genzyme Corporation.

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## Blood Components to Achieve Hemostasis for Surgery and Invasive Procedures

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Blood components are frequently transfused to patients undergoing invasive procedures and surgery. As illustrated by the cases below, decisions regarding transfusion before invasive procedures can be complex.

### Case 1

A 49-year-old man is transferred to your hospital because of fever, altered mental status, and thrombocytopenia (platelet count 35,000/ $\mu$ L). Because the diagnosis of thrombotic thrombocytopenic purpura (TTP) was considered by the referring institution, neither the referring physicians nor your clinical team want to administer platelet transfusions. Although a diagnostic lumbar puncture (LP) was considered important by the referring hospital, the platelet count was considered too low to safely perform an LP and so it was not done. You are called to organize plasma exchange, not only to treat the patient, but also to produce a higher platelet count so that an LP can be safely performed. *Comment: There is absolutely no evidence that a diagnostically important LP should be delayed because of a platelet count of 35,000/ $\mu$ L. In fact, there is evidence demonstrating that an LP is safe at this level of thrombocytopenia (discussed below). You recommend that the LP be done without administration of platelets. The procedure results in no hemorrhagic complication. The results of the LP demonstrate acute bacterial meningitis, the diagnosis of which was unfortunately delayed by the failure to do an important diagnostic test in a patient with fever and altered mental status. The patient does not have TTP.*

### Case 2

Following an extended period of endotracheal intubation, a 60-year-old man, currently in the intensive care unit (ICU), needs a tracheotomy tube and feeding gastrostomy tube placed at the bedside. The platelet count is 40,000/ $\mu$ L and the international normalized ratio (INR) is 1.3. The patient has either medication-related or immune-mediated consumptive thrombocytopenia and does not respond to platelet transfusions. He has recently

received intravenous immunoglobulin. You are told that the surgeon “absolutely refuses” to do the procedure without the prior infusion of four doses of platelets (equivalent to 24 units) with an additional two doses available to “run in” during the procedure. You are also supporting the transfusion needs of a trauma patient (gunshot wound) currently in the operating room (OR); another OR patient who is receiving a heart transplant; and a patient who is scheduled for an aortic aneurysm repair later this evening. You have 12 doses of platelets available and inform the ICU team that you cannot commit six doses of platelets to a bedside tracheotomy-gastrostomy case. The medical ICU team replies that your decision will mean that the patient will not receive his urgently needed tracheotomy and they request that you come up to the floor to explain to a concerned family why you are denying the patient his essential care. *Comment: There is no evidence that the request for preprocedure platelets is medically justified. The use of infusions “run in” during the procedure is largely theatrical. You are correct to manage a limited inventory of platelets by assigning them to active surgical patients who are more likely to benefit from platelet transfusions. The response of the medical ICU team, who attempt to twist the dynamic implying that you are denying the patient essential care, is completely inappropriate. Nevertheless, this sort of attempted coercion occurs commonly. You discuss the issue with the individuals assigned to do the tracheotomy and review with them the published risks on bleeding (described below). Weighing the risks and benefits, they decide to proceed with the bedside tracheotomy and gastrostomy without any preprocedure blood infusions. The procedure goes smoothly with an estimated blood loss of <10 mL.*

### Case 3

A previously healthy 64-year-old man with pneumonia and sepsis requires placement of a central line for hemodynamic monitoring and vascular access. The patient has an INR of 1.6. Because of hypoxia, decreased urine output, and presumed decreased left ventricular performance, circulatory overload is a concern. Despite this, the presiding house-staff physician requests 4 units of fresh frozen plasma (FFP) to “correct the coagulopathy” before central line placement. When you question this decision, the house-staff member acknowledges that he

is “nervous” about placing the line and that “he would feel more comfortable if the patient got FFP first.” *Comment: There is no evidence that preprocedure FFP will benefit this patient undergoing central line placement. Transfusion of this patient, for whom volume overload is a legitimate concern, in order to increase the comfort level of the physician is not appropriate.*

## Current Challenges

As illustrated by the cases presented above, three basic challenges confront our understanding of the proper transfusion support of patients undergoing surgery or invasive procedures. First, there is no consensus on what extent of bleeding should be prevented or should demand treatment. For example, no clinician would transfuse a patient to prevent or to “treat” a 2-cm superficial skin hematoma at the site of insertion of a central venous catheter. While such procedure-related bleeding is not desirable, it is acceptable and represents largely a temporary cosmetic adverse event of little consequence. In contrast, all clinicians would wish to avoid a hemothorax resulting in a collapsed lung, transfusion of 4 units of Red Blood Cells (RBCs), and placement of a chest tube for drainage. The challenge is to understand where the degree of reasonable and acceptable bleeding ends and where the extent of excessive bleeding begins. How much bleeding is acceptable differs for patients depending upon their entire medical status, the nature and urgency of the procedure, and the expected benefit to be gained. The amount of acceptable bleeding differs for each anatomic site. Opinions about excessive bleeding are also influenced by each clinician’s experience and expectations.

A second challenge in transfusion support is the contrast between the use of blood components for *prevention* of bleeding and the use of blood therapies for the *treatment* of bleeding. Although transfusions given as therapy to bleeding patients have an unquestionable rationale, the same cannot be said for transfusions given to prevent bleeding before surgery or invasive procedures. Blood components given before an invasive procedure are likely to benefit patients who have severe derangements of hemostasis or who offer a clear history of bleeding complications following similar procedures. However, blood component therapy given to patients before procedures solely on the basis of abnormal laboratory tests is of uncertain value. Indeed, there is scant medical evidence, if any, that preprocedure therapies are of any benefit when given to patients with mild to moderate abnormalities of laboratory tests of hemostasis. There is also no evidence that a prophylactic strategy is superior to a treatment-of-bleeding strategy. From a risk-to-benefit perspective, prophylactic transfusion strategies can verge on the absurd. Most clinicians have witnessed patients receiving 10 units of platelets and plasma in an attempt to avoid the potential loss of one-tenth of one unit of blood, a hemorrhage that would not have required treatment had it occurred. Nevertheless, prophylactic infusion of blood components is an extremely common medical practice. If indeed prophylactic treatments are of no value for most patients, and if such

treatments cause their own complications in a fraction of recipients, then large numbers of individuals are suffering adverse consequences of such treatments with no attendant benefit.

A third challenge in the area of procedure-related bleeding is to understand the relative value of blood component therapies compared with other agents that promote hemostasis. Although transfusions of platelets or FFP are often used, some patients would fare better were they to receive topical hemostatic agents, medications such as fibrinolytic inhibitors, or ligation of a bleeding vessel by a well-placed suture.

Thus, the decision-making for each patient should consider the following three questions: 1) What extent of bleeding is acceptable? 2) What is the evidence to suggest that any treatment should be given before the procedure? and 3) What is most appropriate therapy to achieve hemostasis? An overview of these considerations is shown in Fig 38-1.

## Preprocedure Blood Components as Prophylaxis Against Bleeding

The last decade has seen explosive growth in the number and variety of invasive procedures performed outside of the OR. In fact, far more invasive procedures are performed outside the operating room than within the OR. Many patients who undergo invasive procedures will have abnormalities on routine laboratory tests of hemostasis. It is very common, although medically unproven, for clinicians to administer blood components to such patients in the expectation of reducing the risk or severity of procedure-related bleeding. The decision to transfuse blood components as prophylaxis before a bedside procedure is subject to three major criticisms.

### Mild-to-Moderate Abnormalities of Common Laboratory Tests Do Not Identify Patients at Increased Risk of Procedure-Related Bleeding

In an excellent review of hemostasis during surgery,<sup>1</sup> Kitchens summarizes the predictive value of coagulation screening tests in three large survey studies representing nearly 4500 patients.<sup>2-4</sup> These studies found that, in the absence of any history of bleeding, the positive predictive value of an abnormal coagulation screening test as a means to forecast bleeding was only 3%. Thus, the great majority of patients (97%) with abnormal coagulation screening tests will have no increased bleeding at surgery. In a systematic review of the published evidence that a prolonged INR would predict bleeding at the time of an invasive procedure, Segal and Dzik<sup>5</sup> found no evidence to conclude that abnormal preprocedure test results should be used as an indication to transfuse blood components. A similar conclusion was drawn by Holland and Sarode.<sup>6</sup>

A common clinical error is to declare that a patient has a coagulopathy because the patient has an abnormal INR or activated partial thromboplastin time (aPTT). This error is epidemic and patients are routinely referred to as “coagulopathic” based

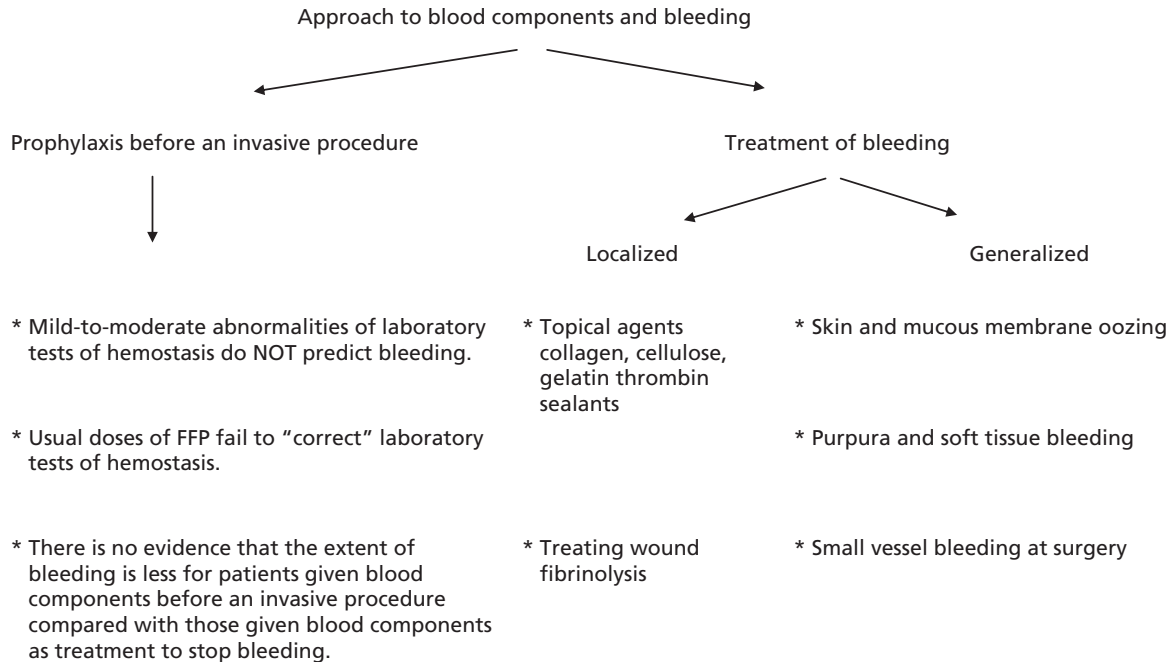
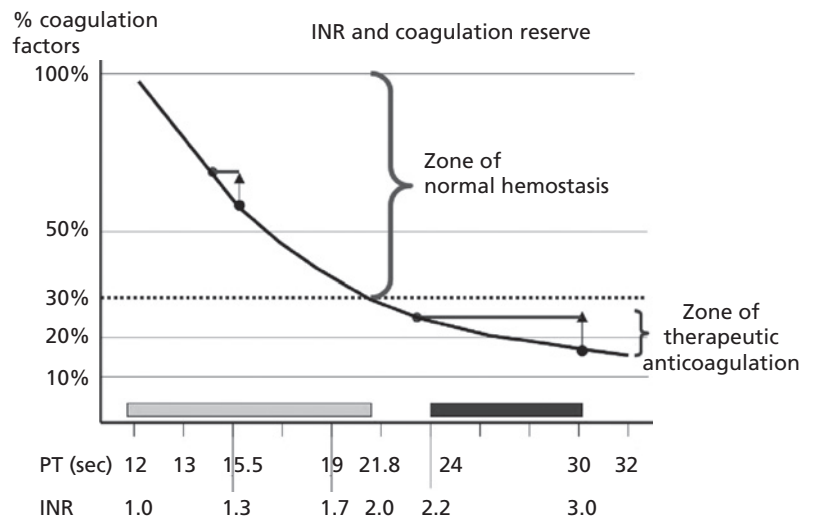


**Figure 38-1.** Overview of blood support for surgery and invasive procedures.

**Figure 38-2.** The nonlinear relationship between the concentration of coagulation factors and coagulation test results. The concentration of factors (y-axis) is shown as a function of the international normalized ratio (INR) or prothrombin time (PT, in seconds). In general, a concentration of 30% is adequate for biologic hemostasis. Note that when starting at an elevated INR, a small increase in the concentration of factors will have a large impact on the measured INR. In contrast, an equivalent rise in the concentration of factors will have a negligible effect on mildly elevated INR values. The curve is a generalized view and will vary for individual patients.



solely on an elevated laboratory test. Clinicians will claim that the indication to transfuse blood was “to correct the patient’s coagulopathy.” Each of these commonly expressed viewpoints fails to consider evidence that an abnormal laboratory test does not, by itself, imply a breakdown in physiologic hemostasis. Indeed, mild to moderate prolongations of the INR or aPTT are completely consistent with normal hemostasis. This is because of the physiologic reserve of coagulation factors and the fact that the laboratory tests are designed to give results outside the range of normal *before* factor deficiencies reach levels of significant physiologic depletion.<sup>7</sup> See Fig 38-2.

Six aspects of the INR and aPTT make these tests poor predictors of hemorrhage.

1. *The relationship between coagulation factors and the prothrombin time (PT) and aPTT is nonlinear.* The exponential relationship between the concentration of clotting factors and the test result is shown in Fig 38-2. This relationship may not be fully appreciated by those who expect a simple dose-response effect from infusion of clotting factors.

2. *Mildly abnormal test results occur among patients with biologically normal coagulation.* In the same way that physiologically adequate tissue oxygenation does not require that the



hemoglobin concentration be in the normal range, physiologic hemostasis does not require that the INR or aPTT value be in the normal range.

**3. INR and aPTT tests overestimate deficiencies in the upper limb of the cascade and underestimate deficiencies in the lower limb.** The INR assay is dominated by the level of Factor VII in the sample. Patients who have mildly reduced levels of Factor VII that are well within the range for adequate hemostasis and who have normal levels of other factors, will have a prolonged INR result. Reduced, but adequate, levels of Factor VII are very common in hospitalized patients because of decreased hepatic synthesis, vitamin K depletion, antibiotics, subclinical impairment of the enterohepatic circulation, and catabolic states with decreased protein synthesis. A very common flaw in the literature is to equate correction of the INR with correction of a hemostatic defect. This misunderstanding has given undo merit to “treatments” that elevate the Factor VII level and produce a dramatic effect on the INR regardless of whether they improve physiologic hemostasis. The same principle is true for the aPTT.

**4. The tests overestimate the extent of coagulation factor depletion if more than one factor is reduced.** Burns et al<sup>8</sup> observed the following in a series of clever in-vitro mixing studies: When plasma containing 50% activity of a single clotting factor was mixed with an equal volume of normal plasma (yielding a mixture with 75% activity of the single deficient factor), the INR and aPTT of the mixture was normal. However, when two plasma samples, each deficient in different factors, were combined with normal plasma samples such that the resulting mixture contained 75% activity of two coagulation factors and 100% of the rest, the resulting INR and aPTT values were prolonged. Thus, for patients with multiple mild factor deficiencies—such as those with liver disease or vitamin K depletion—coagulation tests are prolonged out of proportion to the degree of hemostatic defect that would be assumed based on inherited single-factor deficiencies. With multiple mild deficiencies, patients with factor levels well within the range of normal will show abnormal test results.

**5. Whereas the INR and aPTT are useful tests to analyze the defect in patients with disorders of fibrin formation, neither test was designed to predict bleeding.** Given that clinical hemostasis depends on the complex interrelationships between the blood vessel wall, cellular elements of the blood, platelet number and function, fibrin formation, and fibrinolysis, it is not surprising that the INR and aPTT prove unable to predict who will bleed during surgery. The uncertainty that attends any invasive procedure is stressful for clinicians. This stress coupled with the understandable strong desire to prevent bleeding complications may have seduced clinicians into conferring upon these laboratory tests the power to foresee the future—a power they never have had, and never will.

**6. Most important, these tests are insufficient to assess global hemostasis.** The INR and aPTT focus on fibrin formation in vitro using a highly artificial assay, rabbit brain extracts, synthetic phospholipids, and without the contribution from biologically important molecules such as protein C and thrombomodulin.

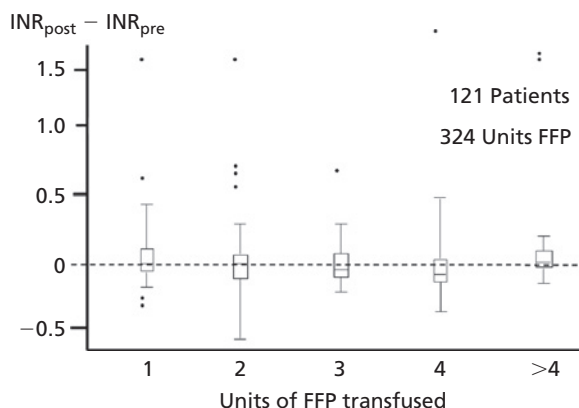


**Figure 38-3.** “Box-and-whiskers” plot of the median change in international normalized ratio (INR) resulting from transfusion of Fresh Frozen Plasma (FFP). The change in INR measured within 8 hours of transfusion of FFP is shown. All patients had INR values  $<1.7$  before transfusion. Used with permission from Abdel-Wahab.<sup>10</sup>

This can lead to incorrect conclusions regarding hemostasis. For example, Mannucci et al<sup>9</sup> have shown that thrombin generation in patients with cirrhosis is incorrectly measured by standard INR and aPTT tests.

### FFP Given Before a Procedure Fails to Correct the Abnormal Coagulation Test Result

For patients with mild to moderate prolongation of the INR or aPTT, there is ample evidence to demonstrate that routine doses of FFP fail to “normalize” the coagulation test. This fact does not prove, by itself, that such transfusions have no value. Rather, the observed findings challenge the commonly expressed opinion that the indication for preprocedure FFP is “to correct” the “coagulopathy” as demonstrated by an elevated INR. Abdel-Wahab and colleagues<sup>10</sup> reported the effect of FFP on the INR in 121 patients transfused with 324 units of FFP. All patients had INR values ranging from 1.1 to 1.85 before transfusion and repeat INR values measured within 8 hours of receiving 1 to 6 units of FFP. Transfusion of FFP resulted in normalization of INR values in  $<1\%$  of patients and decreased the INR value half-way to normal in 15% of patients. The median decrease in INR as a result of transfusion was a negligible 0.07. See Fig 38-3.

Holland and Brooks<sup>11</sup> extended these findings to a larger cohort of adult and pediatric patients treated at the University of Oklahoma. They observed that transfusion of FFP had no effect on the INR values for patients with INRs in the range of 1.7. At higher levels of INR before transfusion, FFP exerted an effect. The effect of a unit of FFP on the INR was linear ( $r^2 = 0.82$ ) for INR values above 1.7 and was represented by an INR change of 0.37 to 0.47. Thus, for a patient with an INR equal to 3, each unit of FFP would be expected to reduce the INR by 0.64. Other studies have also found that FFP fails to normalize laboratory tests.<sup>12-17</sup>

The effect of FFP transfusion on the INR is seen in Fig 38-2. Because the volume of distribution of different

coagulation factors is larger than the intravascular space, 2 units of FFP would be expected to increase the concentration of coagulation factors by 5% to 15%. When the pretransfusion INR is below 1.7, a 10% increase in coagulation factors will have a trivial effect on the measured PT. This fact leads to the following common sequence of events. Because a patient's INR exceeds the local threshold value, FFP is infused and a bedside procedure is performed without checking for any effect of transfusion on the INR. The procedure goes smoothly and the successful outcome is attributed to the transfusion. However, had the INR been measured after the FFP infusion, the clinician would have observed that the posttreatment INR was still above the clinician's own personal threshold. This simple finding combined with the successful procedure outcome makes two points: first, that the treatment failed to "correct" the abnormality; and second, that the procedure was safely performed despite an INR value that was above the clinician's own personal threshold.

*There is no evidence that prophylactic transfusions given before an invasive procedure will limit the extent of bleeding more effectively than therapeutic transfusions given after a procedure.* Kitchen's review noted that only 3% of patients with abnormal coagulation screening tests actually experience surgery-related bleeding. If one were to accept the undocumented opinion that 4 units of FFP and 6 units of platelets given before a procedure would actually decrease the extent of bleeding at the time of the procedure, then 100 candidate patients with abnormal tests would collectively require 1000 units of components as preprocedure prophylaxis. Of these 100 patients, 97 would receive transfusion unnecessarily, encountering a fixed transfusion risk for no benefit. Alternatively, if no preprocedure products were infused, then three patients would experience bleeding that could then be treated with blood components. For the same resource, each one of these three patients would have available as many as 133 units of FFP and 200 units of platelets per patient, representing a quantity of blood support that would certainly be more than adequate to treat bleeding. Thus, from the perspective of both the patient who bleeds and the patient who does not bleed, it would seem that treating well the patient who is actually bleeding is more valuable than an approach that transfuses the vast majority of blood components to individuals who do not need them.

### Toxicity Resulting from Preprocedure Transfusions

If the benefit from prophylactic transfusions is negligible, then even small transfusion risks drive risk-to-benefit considerations away from prophylactic transfusion. Recent studies highlight the susceptibility of ICU patients to toxicities of transfusion. These patients frequently undergo bedside procedures and receive preprocedure transfusions. Khan et al<sup>18</sup> published an important paper documenting that FFP and platelet transfusions are associated with the development of acute lung injury in ill medical patients. They studied 841 consecutive critically ill patients admitted to intensive care at the Mayo Clinic. Of these, 298 (35%) were transfused. Acute lung injury developed more

commonly (25% vs 18%,  $p = 0.025$ ) in transfused patients. Transfused patients remained two-fold more likely to develop acute lung injury than nontransfused patients even when other risk factors for lung injury were accounted for. Of interest, the risk of lung injury was associated with transfusion of FFP or platelets rather than RBCs. Because other studies<sup>19</sup> have found that the most common indication for FFP outside of surgery was prophylaxis before an invasive procedure, the findings of Khan et al underscore the potential adverse effects on critically ill patients from unnecessary exposure to FFP and platelets.

A follow-up study from the same medical center confirmed concerns that FFP and platelet transfusions were associated with acute lung injury in intensive care patients. Gajic et al<sup>20</sup> reported on 901 transfused patients. Of these, an astounding 74 (8%) developed objective signs of acute lung injury within 6 hours of transfusion. When adjusted for patient characteristics, the risk of transfusion-related acute lung injury (TRALI) was significantly higher for each of the following blood donor characteristics: female donor, history of multiple pregnancies, positive test for granulocyte or HLA antibodies, and concentration of phosphatidylcholine in the product. This paper suggests that TRALI is far more common among intensive care patients than has been previously recognized. The high frequency of TRALI in susceptible patients should, by itself, call for a reappraisal of the risk-to-benefit ratio surrounding the transfusion of blood components to nonbleeding patients.

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## Preprocedure Bleeding Risk for Specific Bedside Procedures

### Central Venous Catheter Insertion

Serious complications of central venous catheter placement are rare and include pneumothorax, air embolus, and hemothorax.<sup>21</sup> Significant bleeding following subclavian vein catheterization occurs in patients with perfectly normal hemostasis and results from inadvertent laceration of the subclavian artery.<sup>22</sup> Among patients with advanced liver disease, the insertion of a central venous catheter for transvenous hepatic biopsy provides an excellent model to demonstrate the failure of abnormal coagulation tests to predict bleeding complications at the time of central line placement. Goldfarb and Lebec<sup>23</sup> reported results on central line insertions in 1000 consecutive patients with liver disease, all of whom had abnormalities of laboratory coagulation tests. Only one patient experienced a significant hematoma. Foster et al<sup>24</sup> performed central line insertions on 259 patients with advanced liver disease awaiting transplantation. All patients had serious laboratory derangements including a mean platelet count of 49,000/ $\mu$ L, aPTT equal to 97 seconds, and INR mean of 2.0. No preprocedure products were given and no important bleeding complications occurred.

In an interesting report by Petersen et al,<sup>25</sup> 516 consecutive patients received internal jugular cannulations before cardiac surgery. Of these, 252 (49%) were deliberately fully

anticoagulated with heparin before surgery with a target aPTT of 1.5 times control. Central lines were placed while the patients were anticoagulated. For another 264 patients, anticoagulation was reversed before line insertion. An observer, unaware of the anticoagulation status of each patient, assessed the insertion site. Of the 22 hematomas observed, 13 were found in anticoagulated patients and nine were in nonanticoagulated persons. None of the patients with hematomas required treatment beyond topical care. Of note, among 22 episodes of inadvertent puncture of the carotid artery, the incidence of subcutaneous hematoma formation was the same in both the anticoagulated patients (four of 12 patients with carotid puncture) and the nonanticoagulated patients (three of 10 patients with carotid puncture).

Fisher et al<sup>26</sup> reported results among 658 cannulations that were evenly balanced among internal jugular and subclavian vein insertions. All patients had advanced liver disease and abnormal coagulation tests. The median INR was 2.4 (range, 1-16) and the median platelet count was 81,000/ $\mu$ L (range, 9000-1,088,000/ $\mu$ L). In 580 cases, the INR was  $>1.5$ ; in 531 cases the platelet count was  $<150,000/\mu$ L; and in 453 cases both abnormalities were present. Preprocedure FFP or platelets were not given routinely. One patient, with near normal coagulation tests, had a bleeding complication attributed to technical mishap. Superficial skin hematomas and mild oozing at the puncture site were observed. Logistic regression analysis identified INR values  $>5.0$  ( $n = 137$ ) or platelet counts  $<50,000/\mu$ L ( $n = 146$ ) as statistically associated with a higher incidence of superficial hematomas or site oozing, respectively. The greatest predictor (odds ratio, 8.0) of a procedure-related hematoma was a failed guidewire attempt ( $n = 54$ ). This paper underscored the fact that technical mishaps remain the dominant cause of hematoma formation, and that bleeding complications other than skin hematoma or oozing are rare regardless of the coagulation test results. The paper suggests that patients with an INR  $>5$  or a platelet count  $<50,000/\mu$ L are at a higher risk for simple skin hematoma or oozing following central line placement. Similar studies documenting that

abnormal screening tests are not an indication for preprocedure FFP or platelet transfusions were reported by others.<sup>27-29</sup>

## Liver Biopsy

Closed liver biopsy has generally been considered one of the higher risk bedside invasive procedures. Several features may account for this reputation: patients with liver disease often have multiple derangements of coagulation tests and are known to have truly impaired global hemostasis; biopsies are performed without the aid of direct pressure on the wound site; patients undergoing biopsy may have vascular malignancies. Indeed, sensitive studies using ultrasound analysis have detected small intrahepatic hematomas in as many as one-quarter of the patients who undergo a liver biopsy.<sup>30</sup> For these reasons, there is a strong desire to identify which patients are at increased risk for bleeding. Some programs will not perform closed biopsies on patients with certain locally determined abnormalities of hemostatic screening tests. These patients may receive an open surgical biopsy, a transjugular biopsy, a laparoscopic biopsy,<sup>31</sup> or a biopsy followed by gelfoam plugging.<sup>32</sup>

More than 25 years ago, Ewe et al<sup>33</sup> performed an informative and unique study in which patients underwent liver biopsy using a laparoscope. Following the procedure, the investigators left the laparoscope in place and measured the duration of bleeding from the site of the biopsy. Among 200 consecutive patients with a variety of nonmalignant liver disorders, most patients had at least one abnormal coagulation test but none were treated with blood components before the procedure. The study showed that there was no correlation between the length of time the liver bled after puncture and the preprocedure laboratory values. See Fig 38-4.

Terjung et al<sup>34</sup> reviewed bleeding outcomes among 629 percutaneous liver biopsies performed in 574 patients. No patient had a severe derangement of clotting tests, but some patients had INR values as high as 1.4 and platelet counts below 60,000/ $\mu$ L. Preprocedure transfusions were only rarely given (4%). Bleeding

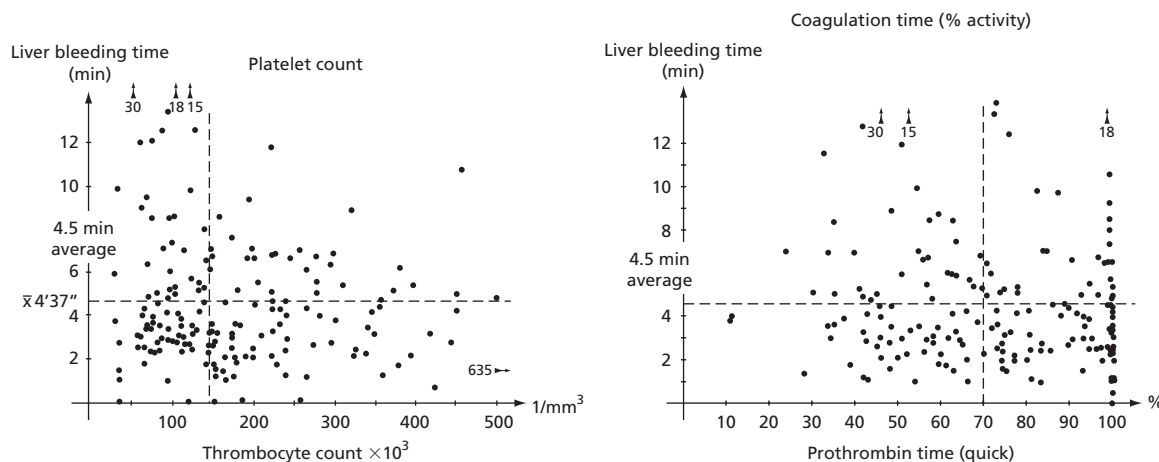


**Figure 38-4.** Lack of correlation between bleeding from the site of liver biopsy and preprocedure coagulation test or platelet count. The duration of bleeding from the site of puncture of the liver (y-axis) is plotted as a function of the preprocedure platelet count (left-hand panel) or the preprocedure coagulation test (expressed as % activity, right-hand panel). There is no correlation between preprocedure laboratory test results and the duration of bleeding. Used with permission from Ewe.<sup>33</sup>

was defined as: “clinically overt” ( $n = 10$ , 1.6%); a postprocedure decline in hemoglobin  $>2\text{ g/dL}$  ( $n = 45$ , 7.1%); or a hematoma detected after the procedure among 292 patients receiving ultrasound ( $n = 31$ , 11%). Using multiple logistic regression analysis, no correlation was found between bleeding risk and the mild to moderate abnormalities in the preprocedure INR, aPTT, platelet count, or bleeding time.

In a prospective study, Makris et al<sup>35</sup> measured INR, aPTT, thrombin time, fibrinogen, bleeding time, and platelet count in 104 patients before liver biopsy. All patients underwent computed tomography scanning 24 hours after the procedure to assess bleeding. More than 50% of patients had one or more abnormalities of laboratory screening test results. INR values ranged as high as 2.0 and platelet counts were as low as 50,000/ $\mu\text{L}$ . Only two patients demonstrated procedure-related bleeding and both of these had *normal* preprocedure laboratory test results.

Other studies examined the role of preprocedure laboratory testing or preprocedure infusion of blood components to prevent bleeding. All studies (except one) found no evidence for the predictive value of screening tests or the clinical value of preprocedure blood component prophylaxis.<sup>36-40</sup> One study suggested that a prolonged preprocedure skin bleeding time assay was associated with bleeding after biopsy, but this small study involved only nine patients with bleeding, three of whom had received marrow transplants.<sup>41</sup>

Given the strong (albeit unfounded) reliance on the INR as a key measure of hemostasis in liver disease and given the dependence of the INR test on Factor VII, it is not surprising that investigators might explore the role of recombinant Factor VIIa given before liver biopsy. Jeffers et al<sup>42</sup> studied 63 patients with a mean INR of 2.0 who received recombinant Factor VIIa in doses ranging from 5 to 120  $\mu\text{g/kg}$  10 minutes before biopsy. Hemostasis at 10 minutes failed in 30% of patients, most of whom were given a postprocedure dose of 80  $\mu\text{g/mL}$ . No toxicity was observed. This study unfortunately lacked a control group and no follow-up testing for hematoma formation was performed. Although the ability of recombinant Factor VIIa to improve the INR in patients with liver disease is undeniable, the fundamental question is whether preprocedure treatment with it is superior to treatment with FFP or to no treatment at all. This would be best answered in a prospective, randomized, controlled clinical trial. The evidence for and against the use of recombinant Factor VIIa for patients without hemophilia was reviewed in a recent Cochrane report.<sup>43</sup>

### Thoracentesis and Paracentesis

Given the lack of evidence in favor of preprocedure transfusions for central venous catheter placement or for closed liver biopsy, it is not surprising that published evidence also fails to support the use of preprocedure prophylaxis for less invasive procedures such as thoracentesis or paracentesis. McVay and Toy<sup>44</sup> reported a retrospective review of outcomes in 608 consecutive procedures (391 paracenteses, 207 thoracenteses, and 10 both procedures). Patients were not transfused before the procedure.

No patient had serious bleeding. The proportion of procedures that were accompanied by a decline in hemoglobin was the same in the group with normal INR/aPTT values as in the group with abnormal INR/aPTT values. A decline in hemoglobin following the procedure also did not correlate with the preprocedure platelet count. The change in hemoglobin was not different among 87 patients with both thrombocytopenia and prolonged coagulation times compared to those with normal values.

Bleeding following paracentesis was examined by Webster et al,<sup>45</sup> who reported a retrospective analysis of 179 outpatients. Four patients developed intra-abdominal or abdominal wall bleeding that required a transfusion of RBCs. The INR values of these four were indistinguishable from those of the 175 patients who did not bleed.

### Gastrointestinal Endoscopy and Biopsy

The use of aspirin or nonsteroidal anti-inflammatory drugs (NSAIDs) and procedure-related bleeding was addressed by Shiffman et al.<sup>46</sup> Of 694 patients who underwent either upper endoscopy with biopsy or colonoscopy with biopsy or polypectomy, half had recently taken either aspirin or NSAIDs. Four patients had serious bleeding requiring hospitalization or treatment—two in the group taking NSAIDs/aspirin and two in the group not taking these medications. After colonoscopy, minor bleeding requiring no therapy was significantly more common in the group taking NSAIDs (6%) than among those taking no medication (2%) (odds ratio, 3).

### Procedures on the Upper Airway, Bronchoscopy, and Transbronchial Lung Biopsy

Special care to provide good hemostasis is prudent when procedures are being performed on the oropharynx, trachea, or bronchi, given that excessive bleeding can be rapidly fatal. Tonsillectomy was an early subject for studies assessing preoperative screening. As with other surgical procedures, the predictive value of an isolated abnormal coagulation screening test for bleeding at tonsillectomy or adenoidectomy is very poor. The majority of patients who bleed during these procedures have normal presurgical tests.<sup>47,48</sup>

Tracheotomy is commonly performed in very ill patients in intensive care, many of whom may have abnormal laboratory tests for coagulation. Auzinger et al<sup>49</sup> partially addressed the approach to such patients in a prospective study of procedure-related bleeding in a cohort of 60 patients with severe liver disease undergoing percutaneous tracheotomy. Patients ranged in age from 18 to 60 years of age and had high severity of illness scores. All but two patients had derangements of laboratory coagulation tests or platelet count. Coagulation abnormalities could not be corrected in 25 patients and 13 patients had platelet counts  $<30,000/\mu\text{L}$ . Only one patient had excessive bleeding (defined as  $>150\text{ mL}$ ), which was external (extratracheal). No patient required open surgical revision. Kluge et al<sup>50</sup> reviewed a 6-year experience with tracheotomy performed in 42 patients with thrombocytopenia. The mean platelet count was 26,000/ $\mu\text{L}$ .

Two patients (<5%) demonstrated excess bleeding. Beiderlinden and colleagues<sup>51</sup> found a low incidence of procedure-related bleeding in 136 patients of whom 18 were known to have a significant coagulopathy. Similar results were found by Ben Nun et al<sup>52</sup> who reported the absence of significant procedure-related bleeding in 157 patients of whom 12 had coagulation abnormalities that could not be normalized despite factor replacement or cessation of anticoagulation. Although these studies all include small numbers of patients with abnormal coagulation tests and do not exclude the possibility that bleeding complications might be slightly higher among patients with measured defects in coagulation, the publications taken together certainly refute the notion that mild to moderate prolongation of the INR or aPTT or mild to moderate thrombocytopenia represent absolute contraindications to safe tracheotomy.

Bronchoscopy and biopsy was directly addressed in an early study by Kozak and Brath.<sup>53</sup> They reviewed 274 patients undergoing 305 fiberoptic bronchoscopy and biopsy procedures at a tertiary care institution. Abnormal coagulation screening tests were found in 10% (n = 28) of patients. Overall, 35 patients bled, and 32 of these had normal preprocedure laboratory values. Three patients had severe bleeding and each of them had normal preprocedure test results. Although the authors noted no utility for prebronchoscopy tests of hemostasis, the number of patients with defined hemostatic abnormalities was too few to develop risk ratios with small confidence intervals (CIs). Further evidence that preprocedure coagulation tests are not a suitable guideline for preprocedure transfusion comes from the work of Diette et al.<sup>54</sup> They reported a retrospective review of 720 fiberoptic bronchoscopies performed over a 1-year period at Johns Hopkins. Using multivariate analysis, they found that bleeding did not correlate with coagulation parameters or platelet count, and that performing a transbronchial biopsy did not independently increase the risk of bleeding. Postprocedure hemoptysis and bleeding (>25 mL) were associated with prior lung transplant (odds ratio, 3.4; 95% CI, 1.3-8.6) and long procedure time (odds ratio, 2.5; 95% CI, 1.2-5.0). The higher incidence of bleeding among lung transplant recipients may relate to anatomic causes such as fragile tissue from poor nutrition or chronic exposure to steroids, mucositis from immune suppression and superinfection, or elevated pulmonary vascular pressures. Bjortuft et al<sup>55</sup> monitored blood loss following 105 consecutive transbronchial lung biopsies and found no correlation with the INR, aPTT, bleeding time, or platelet count. Herth et al<sup>56</sup> studied whether aspirin ingestion increased the extent of bleeding at the time of bronchoscopy and transbronchial lung biopsy. Of 1217 patients, 285 were taking aspirin at the time of the procedure. Bleeding complications occurred in 57 patients (4.7%). The incidence of minor (1.8 vs 2.9%), moderate (1.1 vs 1.4%), and severe (0.8 vs 0.9%) bleeding was not statistically different in those who took aspirin and those who did not. This report provides clinical evidence that there is no requirement to discontinue aspirin ingestion before transbronchial biopsy, nor is the history of recent or current aspirin use an appropriate indication for preprocedure

platelet transfusion. Laboratory tests failed to correlate with bleeding in a separate report by Weiss et al.<sup>57</sup>

In an interesting animal study, Brickey et al<sup>58</sup> did transbronchial biopsies in 18 swine who were treated with escalating doses of warfarin. The object of the study was to determine the INR level at which excess bleeding would occur following the invasive procedure. Excess bleeding was defined as  $\geq 100$  mL blood loss among  $\geq 50\%$  of animals. To the surprise of the investigators, the warfarin-treated animals did not develop excessive postprocedure bleeding despite the fact that 11 of the 18 animals had INR levels >7 at the time of the biopsy. Three highly anticoagulated animals died from spontaneous, nonpulmonary hemorrhage from ruptured ovarian cysts. The authors concluded that INR elevation did not correlate with bleeding following transbronchial lung biopsy in this animal model.

### Renal Biopsy

Davis and Chandler<sup>59</sup> examined the predictive value of hemostasis tests obtained before biopsy of renal allografts. It should be noted that a renal allograft is placed in the pelvic area close to the skin surface and is accessible to biopsy. The template bleeding time, INR, aPTT, platelet count, and thromboelastograph results were examined in 120 patients. Bleeding was defined either as  $\geq 4\%$  decline in hematocrit or as ultrasound evidence of a perirenal hematoma. Overall, 21% of patients showed evidence of mild bleeding, two showed evidence of hematoma on ultrasound, and none required transfusion for bleeding. Most patients with bleeding had normal test results before biopsy. A weakness of this study is that only a few patients had moderate to severe abnormalities of coagulation. Procedure-related bleeding among 70 patients with combined liver and kidney disease who underwent transjugular renal biopsy was reported by Jouet et al.<sup>60</sup> The published literature is not sufficient to draw any strong conclusions regarding hemostatic defects and bleeding at the time of renal biopsy. Because patients undergoing a renal biopsy may have platelet dysfunction from renal insufficiency, it would seem that desmopressin might be a very useful agent to administer before biopsy.

### Epidural Anesthesia, Diagnostic Lumbar Puncture, Neurosurgical Procedures

Because bleeding in the closed space of the subarachnoid or epidural region can produce paraparesis or paraplegia following lumbar puncture, avoiding preventable bleeding is more important for lumbar punctures and epidural anesthesia than for most invasive procedures.

Nevertheless, several studies provide evidence that thrombocytopenia is not a major contraindication to diagnostic LP. Howard et al<sup>61,62</sup> reported an extensive experience with >5000 LP procedures performed in 958 children with leukemia. These children received repeat LPs for administration of intrathecal chemotherapy. Despite the fact that platelets in these children may have been dysfunctional because of leukemia or antibiotics, there were few hemorrhagic complications. Some 170 LPs were performed in patients with platelet counts between 10,000 and 20,000/ $\mu$ L;

742 LPs were performed in patients with platelet counts between 20,000 and 50,000/ $\mu$ L; and 858 LPs were performed in patients with platelet counts between 50,000 and 100,000/ $\mu$ L. Platelet transfusions were not given before the procedure. No patient developed spinal hematoma or a clinical bleeding complication. The authors defined a “bloody” LP as one with  $>500$  red cells/ $\mu$ L. In contrast, normal blood has  $\sim 5 \times 10^6$  red cells/ $\mu$ L. Using this very conservative definition of procedure-related bleeding, they noted that the likelihood of finding  $>500$  red cells/ $\mu$ L in the spinal fluid was slightly higher in patients with platelet counts  $<100,000/\mu$ L compared to those with counts  $>100,000/\mu$ L (odds ratio, 1.5; 95% CI, 1.2-1.8). However, among patients with counts  $<100,000/\mu$ L, the likelihood of an LP with  $>500$  red cells/ $\mu$ L did not increase as the platelet count declined to levels as low as 10,000/ $\mu$ L. The authors concluded that a platelet count of 10,000/ $\mu$ L was adequate to perform a routine LP for administration of intrathecal chemotherapy. A smaller but similar retrospective study extended these same conclusions to adults with leukemia. Vavricka et al<sup>63</sup> reviewed records of 195 LPs performed on 66 adult patients (age 18-68) over a 6-year period. No patient developed spinal hematoma or clinical hemorrhagic complications. As found by Howard et al,<sup>61,62</sup> patients with platelet counts  $<100,000/\mu$ L were more likely to have microscopic bleeding ( $>500$  red cells/ $\mu$ L). The authors recommended that prophylactic transfusions be given only if the platelet count were  $<20,000/\mu$ L. These studies provide evidence that an LP, if of diagnostic importance, should not be delayed or withheld because of thrombocytopenia.

Other studies, with smaller numbers of patients, also found no relationship between hemorrhagic complications of LP and the platelet count. For example, Waldman et al<sup>64</sup> used a small (25-gauge) needle to administer morphine via a caudal block to 19 patients with platelet counts  $<50,000/\mu$ L without bleeding complications. Rasmus et al<sup>65</sup> reported that none of 14 women with platelet counts ranging from 15,000/ $\mu$ L to 100,000/ $\mu$ L developed complications from epidural anesthesia given at the time of childbirth. Hew-Wing et al<sup>66</sup> reviewed the literature on the issue of epidurals and thrombocytopenia and could find no case report of a thrombocytopenic patient who developed a hematoma after epidural anesthesia. However, they did identify one report of eight cases of spinal subdural hematoma complicating LP among leukemia patients.<sup>67</sup>

In contrast to thrombocytopenia, the chance of peridural hematoma appears to be increased among patients with full-dose anticoagulation who undergo placement or removal of an epidural catheter for spinal anesthesia. In patients with low-dose anticoagulation undergoing vascular surgery, Rao and El-Etr<sup>68</sup> did not find evidence of epidural hematomas following placement of either an epidural catheter ( $n = 3164$ ) or a subarachnoid catheter ( $n = 847$ ) using a 17-gauge needle. The mean heparin dose was 10,500 units per 24 hours. Catheters were left in the spinal space for 24 hours and were then removed while patients were still being treated with heparin. No patients developed peridural hematomas. In a similar manner, low-dose heparin was not associated with spinal hematoma in over 5000 patients

undergoing spinal or epidural anesthesia.<sup>69</sup> However, with full-dose anticoagulation, the risk may increase. For example, Ruff and Dougherty<sup>70</sup> reported five cases of paraplegia among 342 patients who received a diagnostic LP and then were administered full-dose heparin (exact dose not stated). In addition, the introduction of low-molecular-weight heparin treatment in the United States was accompanied by an observed increase in reported spinal hematomas among patients with epidural catheters, estimated to occur at a rate of 1 in 10,000 patients.<sup>71</sup>

In this author's experience, neurosurgeons are often strong advocates for preprocedure transfusions before invasive procedures. Some neurosurgeons will flatly refuse to perform procedures they believe to be otherwise clinically indicated solely based on mild to moderate abnormalities of the preprocedure laboratory results. Against this position is the report by Schramm et al<sup>72</sup> who reviewed outcomes among 1211 patients having neurosurgical procedures at the Royal Melbourne Hospital in Australia. Consecutive neurosurgical procedures were examined for a period of 1 year to identify preoperative risks for bleeding. The authors tabulated not only clinical features such as the presence of malignancy or renal failure, liver disease, trauma, history of alcohol use, malabsorption, history of prior surgical bleeding, and medications, but also results of laboratory tests including the INR, aPTT, and platelet count. Three groups of surgical procedures were examined: craniotomies ( $n = 675$ ), spinal surgeries ( $n = 400$ ), and other ( $n = 136$ ). The authors found that 17% of preoperative laboratory tests were abnormal. Using logistic regression analysis to identify factors associated with bleeding, they found that neither prolongation of the INR nor depression of the platelet count predicted excessive surgical bleeding. However, two independent predictors of bleeding were found. First, procedure-related bleeding was associated with a small percentage of patients ( $n = 14$ ; 1%) who gave a history of bleeding and who demonstrated a prolonged aPTT (odds ratio = 24; 95% CI, 4-150). These patients may have had von Willebrand disease. Second, procedure-related bleeding was found among a larger number of patients ( $n = 675$ ; 56%) in association with craniotomy regardless of all other factors examined (odds ratio, 10; 95% CI, 2-54).

### Angiography

The increasing use of interventional radiology and cardiology has created a new cohort of patients at risk for arterial bleeding following angiography. Not surprisingly, there is little evidence that preprocedure blood components are indicated for angiographic procedures. Darcy et al<sup>73</sup> prospectively studied 1000 consecutive patients undergoing femoral arterial puncture for diagnostic or therapeutic vascular procedures. Analysis was restricted to patients with INR values  $<1.5$ . The incidence of hematoma formation was the same in patients without any abnormality of the preprocedure INR (9.7%) as those with abnormal results (8.1%). Neither the INR nor the aPTT was predictive of hematoma formation and each test had a  $<10\%$  positive predictive value. However, hematoma formation was more common among patients with platelet counts  $<100,000/\mu$ L, with

the use of catheters larger than 5 Fr, and among patients with a documented history of bleeding disorders.

### **Recommendations**

Prospective, controlled trials in which patients with similar degrees of abnormal hemostasis are randomly assigned to receive or not receive preprocedure blood components are needed to be able to make firm recommendations on the value of prophylactic transfusions before bedside invasive procedures. Given the low incidence of any form of hemorrhagic complications even among patients with known defects, such studies should be designed as multicenter trials in order to accrue a sufficient number of patients to have adequate statistical power. In the absence of such studies, the current literature can serve to suggest some guidelines for the clinical approach to bedside procedures. There is a substantial lack of evidence that mild to moderate abnormalities of the INR or aPTT can be or should be used as an indication to administer preprocedure FFP. Profound thrombocytopenia ( $<20,000/\mu\text{L}$ ), although a poor predictor of bleeding, arises more commonly than prolongation of the INR or aPTT as an independent risk factor for procedure-related bleeding. Indeed, severe thrombocytopenia ( $<20,000/\mu\text{L}$ ) represents not only a more correctable defect but also a greater absolute defect in hemostasis than mild to moderate prolongation of coagulation times. On the basis of the published literature, preprocedure transfusions are not routinely required for patients with an INR  $<3$  accompanied by a platelet count  $>50,000/\mu\text{L}$ ; nor are they required for patients with  $>20,000/\mu\text{L}$  platelets accompanied by an INR  $<1.7$ . For patients with mixed coagulation disorders (for example, patients with platelet counts in the range of 20,000 to 50,000/ $\mu\text{L}$  accompanied by an INR in the range of 1.7 to 3.0) individual decisions regarding the risk of bleeding are needed. These decisions may be influenced by experience of the operator, technical factors, anatomy, and disease-related comorbidities.

When deciding on the clinical approach to a particular patient, clinicians and laboratorians should recognize that any strategy of prophylactic blood transfusions triggered solely by laboratory screening test results is not supported by published evidence. Clinicians must recognize that mild to moderate abnormalities of laboratory screening tests do not necessarily define a clinical defect in hemostasis. In addition, the decision to transfuse blood components for hemostasis is completely defensible when transfusions are given to arrest bleeding, but are difficult to justify as prophylaxis. As is true throughout clinical medicine, the best decisions are made in the context of each patient's full clinical picture rather than on the basis of laboratory values alone.

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### **Transfusion of Blood Components to Treat Bleeding**

The principal question in addressing transfusion therapy for the bleeding patient is to decide whether the bleeding is primarily localized or generalized.

### **Local Treatments for Local Bleeding**

Upon removing a large kitchen knife from a stab wound to the chest and seeing copious blood emerge from the wound, no physician would elect to treat the problem by ignoring the wound and infusing FFP in the antecubital vein. Yet, countless patients with local bleeding are treated this way every day. Patients with bleeding varices, bleeding ulcers, bleeding from central lines, or bleeding postoperatively from surgical sites are frequently given blood component infusions to stop local bleeding, when a direct approach to the site of bleeding would be more effective. Systemic infusion of blood components for local bleeding is most common in patients who have mild abnormalities of coagulation tests, presumably under the unsupported assumption that the local bleeding results from hemostatic defects reflected by these laboratory tests. While systemic infusion of FFP or platelets may contribute to improved hemostasis at the site of tissue injury and bleeding, transfusions are secondary to a direct approach to hemostasis.

Suture ligation of a damaged vessel remains the single most effective hemostatic treatment for localized bleeding and, if technically possible, should always be considered the treatment of choice. For bleeding from vessels that cannot be ligated, a variety of other choices exist including: electrocautery, injection sclerosis of vessels, argon-laser beam coagulation, and direct packing and compression.

### **Topical Agents: Collagen, Cellulose, and Gelatin**

A variety of topical agents are used routinely during surgery to improve hemostasis, especially at sites of bleeding that cannot be approached by ligation. None of these agents are perfect and all rely upon creating a surface for platelet adhesion and fibrin clot formation. Although useful for oozing, they are washed away by vigorous bleeding and are no substitute for suture. The available compounds are sterile and can be left inside the body, where they dissolve. There is very little known toxicity to these agents. Commercially available agents include products based on collagen (Avitene, C.R. Bard, Murray Hill, NJ; FloSeal, Baxter, Deerfield, IL), cellulose (Surgicel, Johnson & Johnson, New Brunswick, NJ), and gelatin (Gelfoam, Pfizer, New York, NY). For more information, the reader is referred to a fine review by Jackson.<sup>74</sup>

### **Topical Sealants**

In some situations, topical application of blood components to the site of bleeding can be therapeutic. A common example is the use of "fibrin sealant." Fibrin sealant consists of the simultaneous application of topical fibrinogen (usually supplied as 1-2 units of cryoprecipitate) plus topical thrombin. Fibrin sealant will not stanch massive bleeding, but it can prove very useful for sites where a suture cannot be placed. Fibrin sealants are also provided commercially. Currently, Tisseel VH (Baxter) is licensed for use in the United States. It consists of separate vials containing either purified human fibrinogen plus aprotinin or purified human thrombin. Fibrin sealants may also be packaged with a

pump used to spray the sealant on a bleeding surface. Fibrin-based sealants have also been incorporated into dressings that may be applied to the site of bleeding to provide not only direct pressure but also local high concentrations of fibrin for clot formation. Fibrin sealant dressings are under active investigation for use by the military and for trauma applications. Fibrin sealant sprays have been used in cardiac or vascular surgery, especially to address bleeding along lines of vascular anastomosis. Liquid fibrin sealants are used in burn care, liver surgery, maxillofacial surgery, neurosurgery, and some orthopedic surgery.<sup>74</sup>

### Topical Thrombin

Topical thrombin of bovine origin is available as a powder, packaged as 5000 units or 20,000 units. It is usually reconstituted in a diluent and applied at concentrations of 1000-2000 units/mL. It may also be applied directly in powdered form to the site of bleeding. Thrombin is also marketed as a liquid in a spray pump bottle. Recently, the Food and Drug Administration approved both a human thrombin (Evithrom, Johnson & Johnson) and a recombinant thrombin (Recothrom, ZymoGenetics, Seattle, WA). The use of bovine-derived thrombin has been associated in a minority of patients with the development of antibodies directed against bovine coagulation proteins in the preparation. These antibodies include Factor V antibodies and thrombin antibodies. In some patients, these antibodies can cross-react with human coagulation proteins, resulting in the development of a coagulation inhibitor. In some of these patients, the inhibitor can be clinically significant and represents a serious adverse effect of bovine thrombin. However, clinically significant inhibitors are very infrequent and this risk of inhibitor formation should be further reduced with the advent of human and recombinant thrombin. Thus, direct thrombin application remains a useful adjunct to topical hemostasis.

### Topical Antifibrinolytics

In surgical cases, such as trauma surgery, some patients may have substantial diffuse oozing with no defined vessel to ligate. For such bleeding, direct packing and closure with subsequent return to the OR for definitive hemostasis can be lifesaving. In addition to simple packing with sterile gauzes, some clinicians believe there is benefit to packing bleeding sites with gauzes made wet with applied thrombin, antifibrinolytics (eg, Amicar, Xanodyne Pharmaceuticals, Cincinnati, OH), or platelets. The author's institution uses gauzes applied with antifibrinolytics in selected patients.

### Treating Wound Fibrinolysis

The serosal cavities of the body are lined by tissues that are rich in fibrinolytic proteins such as tissue plasminogen activator. When blood enters such cavities it undergoes rapid clot lysis. This accounts for the observed finding that blood in the pericardial space, the pleural space, the peritoneal space, or in joint spaces exists as a liquid that can be extracted with a needle. Blood extracted from such cavities will not clot *ex vivo* because it has

already undergone complete clot lysis. An example of this is seen with the unclottable blood collected into canisters from chest tube drains. A common problem after chest surgery is bleeding into the pleural and mediastinal space. In some patients, if blood drainage is incomplete, the local environment surrounding the bleeding wound site can become bathed in fibrinolytic blood. Thus, wound bleeding into intrapleural, intramediastinal, or intra-abdominal sites can result in an unfavorable cycle in which bleeding into the cavity undergoes clotting and lysis and the fibrinolytic process breaks down clots and results in more bleeding. The principal treatment for this condition is removal or drainage of blood from the body cavity, a therapy underscored by the time-honored surgical practice of washing out retained blood. However, when this is not possible, wound fibrinolysis may occur and simple infusion of blood components is not likely to be effective. For these patients, a short course of a systemic antifibrinolytic agents (eg, Amicar) may be beneficial.

### Treatment of Generalized Bleeding

#### Skin and Mucous Membrane Oozing

Visible, annoying, distasteful, frightening, and unsightly skin and mucous-membrane bleeding often demands attention even if not life-threatening. Often a sign of thrombocytopenia or impaired platelet function, skin and mucous-membrane bleeding may also result from drug eruptions, mucositis, burns, or other damage to skin. Platelet transfusions may be given to patients with petechiae under the assumption that skin bleeding is a harbinger of more serious life-threatening bleeding to come. It is difficult to prove or refute this contention, but petechiae or mucosal bleeding in conjunction with thrombocytopenia would seem a far more logical indication for prophylactic platelet transfusion than a platelet count alone. For patients who are refractory to platelet transfusions as a result of alloimmunization, oral or intravenous antifibrinolytics have been used.

#### Purpura and Soft-Tissue Bleeding

Purpura and soft-tissue bleeding may imply a serious hemostatic defect and are nearly always worthy of investigation. An exception is "senile purpura"—a largely cosmetic problem limited to the extensor surfaces of the forearms in elderly patients with visibly fragile skin. Purpura generally suggests an abnormality in fibrin formation and treatment is directed at the underlying defect. When no specific hemostatic abnormalities are found, the cause may prove to be vasculitis, drug purpura, amyloidosis, or even factitious. Vasculitic bleeding can range from dermal bleeding, as seen in palpable purpura, to life-threatening hemorrhage as seen in pulmonary vasculitis. Rare coagulation defects, such as Factor XIII deficiency, dysfibrinogenemias, or conditions with excessive fibrinolysis may result in purpura without striking abnormalities found on routine laboratory tests. Aneurysms and vascular anomalies can sometimes present as confusing cases of unexpected bleeding. Patients with persistent recurrent gastrointestinal bleeding may have Osler-Weber-Rendu disease (hereditary hemorrhagic telangiectasias). In some patients, the



external telangiectasias that provide the clue to the diagnosis are subtle and limited to the oral mucosa. Vitamin C deficiency (scurvy) typically results in gum bleeding and perifollicular petichiae, but more substantial bleeding with large plate-like areas of skin and soft-tissue purpura may also occur. Unrecognized von Willebrand syndrome or platelet storage pool disorders can result in unexpected bleeding, although most patients with serious examples of these conditions will give a history of easy bruising, heavy menstrual bleeding, iron deficiency, or prior surgical hemorrhage.

### Small Vessel Bleeding During Surgery

Diffuse small vessel bleeding during surgery is not uncommon. In many cases, such generalized bleeding is a sign of a recognizable and correctable hemostatic disorder. Anesthesiologists and surgeons caring for patients undergoing major surgery need access to basic coagulation tests with rapid turnaround time. Usually the patient with diffuse oozing at the end of surgery has developed dilutional thrombocytopenia or dilutional depletion of coagulation factors and these conditions can be corrected by transfusion. In other cases, the results of intraoperative coagulation testing point more toward disseminated intravascular coagulation. This condition should be anticipated if the patient has experienced prolonged hypotension with tissue hypoperfusion and ischemia. In this author's experience, a commonly overlooked cause of diffuse small vessel bleeding is fibrinolysis. With hypotension, pressors, and acidosis, patients will release tissue plasminogen activator from vascular sites. The plasmin that is formed localizes preferentially to fibrin. Therefore, even before a patient will demonstrate fibrinogenolysis (with low systemic fibrinogen levels), the patient will first develop fibrinolysis with dissolution of previously formed clot. In this early fibrinolytic response, there is no measurable abnormality of the circulating blood except for the presence of increased concentrations of D-dimers as a sign of fibrin clot dissolution. Indeed, some degree of fibrinolysis is nearly universal in surgery. Thus, the observation in the OR that the patient is oozing from surgical sites that were previously well clotted should be taken as a sign of the possibility of increased clot lysis. Fibrinolysis is not corrected by infusions of FFP, which may only serve to add plasminogen and create more plasmin. If the bleeding is sustained and if there are no contraindications to the use of an antifibrinolytic agent, then a single bolus dose or a loading dose followed by a brief infusion of an antifibrinolytic may prove very useful.

### Conclusion

James Blundell administered the first human blood transfusions in the early years of the 19th century to treat patients who were actively bleeding. When viewed from an evidence-based perspective, little has changed in 200 years. Blood component therapy remains a remarkable lifesaving treatment of unquestionable value in patients with significant bleeding. Transfusion

of blood to bleeding patients rests on solid principles of resuscitation, tissue oxygenation, and the repletion of vital elements of hemostasis. In stark contrast, transfusion given as prophylaxis based on abnormal screening tests offers unfavorable risk-to-benefit and cost-to-benefit ratios.<sup>75</sup> Clinical research using the outcome measure of bleeding should be conducted to refine the indications for blood use at surgery and before invasive procedures. Better tests of hemostasis are needed. In the meantime, many lives will be saved by transfusion given to bleeding patients. In some cases, these transfusions will directly arrest bleeding by replacing depleted elements of hemostasis. In many more cases, transfusion will serve as an exquisite life-support measure while other interventions are used to stop hemorrhage.

### Disclaimer

The author has disclosed no conflicts of interest.

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# Transfusion Therapy in the Care of Trauma and Burn Patients

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In 1915, Lewisohn published the first reports of collecting blood in sodium citrate at Mount Sinai Hospital in New York, and by 1917, the first military blood bank was set up by Major Oswald H. Robertson of the United States Army. What drove the technology in blood transfusion and storage was the demand for transfusion during World War I.<sup>1</sup> Although the first described blood transfusion was performed in the early 19th century, widespread acceptance of transfusion therapy did not occur until Landsteiner described the ABO blood groups. Experience gained during World War I and the Spanish Civil War led to technologies to collect, store, and transfuse blood in emergencies. Bernard Fantus in 1937 opened the first blood bank in the United States at Cook County Hospital in Chicago. The use of the preservative acid-citrate-dextrose was implemented by the end of World War II, further expanding the ability to store blood.<sup>2</sup> During this same period, the importance of resuscitation in the treatment of shock and trauma was recognized and the use of crystalloids began.

The ability to store blood in large quantities was a boon for trauma surgery. The liberal use of group O Rh-negative blood during the Vietnam War significantly reduced mortality.<sup>3</sup> Coupled with rapid transport time, aggressive fluid resuscitation, and early operative intervention for patients not responding to resuscitation, this advance marked the beginning of modern trauma surgery. The lessons learned from wartime experience with the resuscitation of injured patients were applied to the civilian injured, and by the late 1960s, organized prehospital emergency services were rapidly transporting patients to trauma centers.

The use of blood for the resuscitation and treatment of thermally injured patients was advocated as early as the 1920s and 1930s.<sup>4</sup> Blood and blood components have since been part of various treatment regimens and burn formulas for the initial resuscitation of burn patients. The ready availability of blood and blood components has improved survival among elderly patients and patients with associated medical problems who otherwise would not have tolerated a low hemoglobin level. Intraoperative transfusion has

allowed wider, more aggressive débridement procedures, thereby decreasing the number of surgical procedures needed by a patient.

The current indications for transfusion in trauma and burn care recognize the merits of this form of treatment but acknowledge the practical considerations regarding transfusion. The limitations of the blood supply are apparent. In the United States, more than 14 million units of Whole Blood and Red Blood Cells (RBCs) are transfused yearly to approximately 5 million recipients, with most transfusions being administered in the perioperative period. RBCs have a limited shelf life and are discarded after 42 days. In addition, the incidence of transfusion reactions and of the transmission of hepatitis was well documented by the 1970s. The risk of transmission of human immunodeficiency virus or hepatitis C virus are each estimated to be 1 in approximately 2,000,000 units of blood.<sup>5</sup> On a yearly basis, approximately 35 patients die per year of hemolytic transfusion reactions (mostly from ABO-incompatible transfusion), transfusion-related acute lung injury, or bacterial contamination.<sup>6</sup>

Concerns have also been raised about the possible immunosuppressive effects of transfusion, citing associations between transfusion and infections or recurrence of malignancies.<sup>7,8</sup> In addition, it is known that 50% of severely injured patients who survive the immediate posttrauma phase are at increased risk for the development of the systemic inflammatory response syndrome and may be vulnerable to immunomodulatory effects of transfusions. Although a transfusion is still a lifesaving treatment in the appropriate circumstances, the foregoing observations have led to a more judicious use of blood and blood components as the risk-to-benefit considerations are more fully understood. Current blood use in treating trauma and burns, therefore, must combine judgment and careful assessment of the risk-to-benefit ratio.

## Shock

### Definition of Shock

Shock is defined as inadequate organ perfusion and tissue oxygenation. After trauma, the most common type of shock is hemorrhagic. Neurogenic shock after high cervical spinal injuries and septic shock after penetrating trauma also are possible. After burns,

hypovolemic shock is caused by fluid sequestration at the site of injury and in unburned injured tissue as a systemic response to injury and by evaporative loss caused by loss of the dermal barrier.

A critical goal in the care of trauma and burn patients is optimization of oxygen delivery and prevention of tissue hypoxia. Although it would be ideal if there were a simple laboratory value that could identify the need for transfusion to optimize oxygen delivery, such a magic bullet does not exist. The decision to administer transfusion must be based on an understanding of the role of hemoglobin in the definition of oxygen delivery.

Oxygen delivery ( $\text{DO}_2$ ) is the product of arterial oxygen content ( $\text{CaO}_2$ ) and cardiac output (CO):  $\text{DO}_2 = \text{CaO}_2 \times \text{CO}$ . Arterial oxygen content is a measure of both hemoglobin-bound and free oxygen. It is calculated with the following equation:

$$\text{CaO}_2 = (1.34 \times \text{SaO}_2 \times \text{Hb} \times 10) + (0.003 \times \text{PaO}_2)$$

The left term of the equation ( $1.34 \times \text{SaO}_2 \times \text{Hb} \times 10$ ) calculates the amount of oxygen bound to hemoglobin. The constant 1.34 is an estimate of the mean volume of oxygen in milliliters that can be bound to 1 g of fully saturated normal hemoglobin,  $\text{SaO}_2$  is oxygen saturation, and Hb is the hemoglobin in g/dL. Ten is a correction factor converting g/dL to g/L. The right term of the equation ( $0.003 \times \text{PaO}_2$ ) is the estimation of the dissolved component of arterial oxygen content.  $\text{PaO}_2$  is the partial pressure of arterial oxygen and the constant 0.003 is the solubility coefficient of oxygen in blood at normal body temperature and atmospheric pressure. From this equation, it is apparent that dissolved oxygen contributes little to total  $\text{CaO}_2$  at atmospheric pressure. This contribution would be increased with the use of hyperbaric oxygen, which increases the amount of oxygen dissolved in the blood.

Oxygen consumption ( $\text{VO}_2$ ) is a measure of total oxidative metabolism and is a product of the arteriovenous oxygen difference ( $\text{CaO}_2 - \text{CvO}_2$ ) and the cardiac output (Q). In most cases, oxygen consumption is independent of hemoglobin concentration over a wide range of oxygen delivery values; compensation occurs through increases in cardiac output and oxygen uptake by the tissues. The oxygen extraction ratio, the ratio between oxygen consumption and oxygen delivery, is approximately 25% to 30% under normal conditions. However, different tissues have a variable ability to increase oxygen extraction, further increasing tissue oxygen availability in those tissues. The heart is at maximal oxygen extraction under baseline conditions and depends on increased delivery to meet increasing oxygen demands.

It has been shown in experiments in dogs that, in the normovolemic state, cardiac output progressively increases with decreases in hematocrit to a level of  $8.6\% \pm 0.4\%$ , after which time the dogs went into heart failure.<sup>9</sup> This occurs through reductions in blood viscosity and an increase sympathetic nervous activity.<sup>10</sup> The change in blood viscosity decreases total peripheral vascular resistance, increases postcapillary venular flow, and increases venous return.<sup>10</sup> The increased sympathetic activity results in increased cardiac contractility,<sup>11,12</sup> and increased venomotor tone.<sup>10</sup> As the hematocrit decreases from

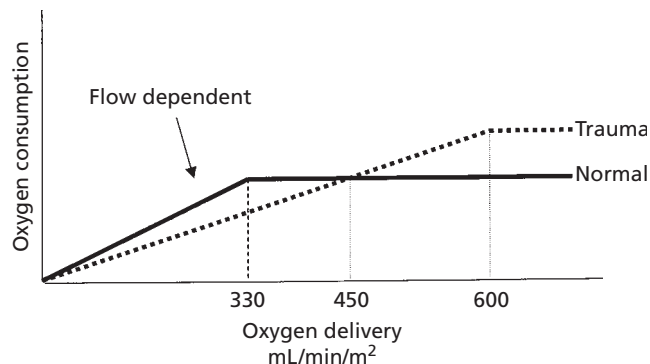


**Figure 39-1.** Unrecognized flow-dependent oxygen consumption ( $\text{VO}_2$ ) under normal conditions and in a trauma state. Critical oxygen delivery ( $\text{DO}_2$ ) in anesthetized adult is 330 mL/minute/ $\text{m}^2$ . The normal oxygen delivery index is 450 mL/minute/ $\text{m}^2$ . The survivor response after normal resuscitation is 600 mL/minute/ $\text{m}^2$ . Used with permission from Moore et al.<sup>13</sup>

the normal value to 30%, oxygen delivery increases to approximately 110% of baseline level.<sup>10</sup> It has been demonstrated in dog models that these compensatory mechanisms allow maintenance of oxygen delivery and uptake until the hematocrit decreases to approximately 10%, after which there is a marked decline.<sup>11</sup>

The relationship between oxygen delivery and oxygen consumption is biphasic (Fig 39-1). At less than a critical level of oxygen delivery, consumption is related to delivery in a linear manner. At greater than critical oxygen delivery, consumption is independent of delivery, the flow-independent portion of the curve.<sup>10</sup> Analysis of patients under anesthesia showed that the critical value of oxygen delivery differentiating flow-dependent and flow-independent oxygen consumption is 333 mL/minute/ $\text{m}^2$ .<sup>14</sup> Shoemaker et al<sup>15</sup> compared survivors with nonsurvivors of elective surgery. They found that nonsurvivors had lower myocardial performance indicators, higher pulmonary artery pressures and pulmonary vascular resistance, and lower oxygen delivery than did the survivors. A study by Boyd et al<sup>16</sup> showed a reduced mortality rate and reduced incidence of postoperative complications if inotropic agents were used to increase cardiac output with a target oxygen delivery of 600 mL/minute/ $\text{m}^2$ .

After major trauma, several factors alter the  $\text{DO}_2/\text{VO}_2$  relation. First,  $\text{VO}_2$  increases to 150 mL/minute/ $\text{m}^2$  to repay ongoing oxygen debts and to support the systemic inflammatory response syndrome. At the same time, peripheral oxygen extraction is impaired, which decreases the slope of the flow-dependent portion of this relation (Fig 39-1). It is possible that a trauma patient with a normal  $\text{DO}_2$  of 450 mL/minute/ $\text{m}^2$  may be on the flow-dependent portion of the curve.<sup>17</sup> Bishop et al<sup>18</sup> found that resuscitating to supranormal values of vital signs, urine output, and pulmonary artery catheter parameters decreased the risk of multiple organ failure and death, but the results of this study have not been duplicated despite numerous attempts.<sup>19-21</sup> In fact, some studies have shown an increase in the mortality of critically ill surgical and medical patients with the use of dobutamine to augment oxygen delivery and consumption parameters.<sup>22</sup> What seems to be a common observation in these studies is

**Table 39-1.** Blood Losses and Classes of Shock Based on Patient's Initial Presentation

|                               | Class I             | Class II       | Class III             | Class IV              |
|-------------------------------|---------------------|----------------|-----------------------|-----------------------|
| Blood loss (mL)               | Up to 750           | 750-1500       | 1500-2000             | >2000                 |
| Blood loss (% blood volume)   | Up to 15            | 15-30          | 30-40                 | >40                   |
| Pulse rate (beats/min)        | <100                | >100           | >120                  | >140                  |
| Blood pressure                | Normal              | Normal         | Decreased             | Decreased             |
| Pulse pressure (mm Hg)        | Normal or Increased | Normal         | Normal                | Normal                |
| Respiratory rate (breath/min) | 14-20               | 20-30          | 30-40                 | >35                   |
| Urine output (mL/hour)        | >30                 | 20-30          | 5-15                  | Negligible            |
| Mental status                 | Slightly anxious    | Mildly anxious | Anxious, confused     | Confused, obtunded    |
| Fluid replacement (3:1 rule)  | Crystalloid         | Crystalloid    | Crystalloid and blood | Crystalloid and blood |

Patient is a 70-kg man.

These guidelines are based on the 3-for-1 rule. This rule derives from the empiric observation that most patients in hemorrhagic shock need as much as 300 mL of electrolyte solution for each 100 mL of blood loss. Applied blindly, these guidelines can result in excessive or inadequate fluid administration.

Used with permission from American College of Surgeons Committee on Trauma.<sup>27</sup>

that achieving adequate or supranormal oxygen delivery serves more as a prognosticator of survival than an endpoint of resuscitation. Trauma patients who do not respond to resuscitation, or to augmentation with inotropes to supranormal hemodynamic parameters, are at higher risk of mortality when compared to the patients who are resuscitated successfully or respond to inotropic support.

In the care of critically ill patients in whom maximal increases in cardiac output have occurred without the necessary improvements in oxygen delivery needed to reach the flow-independent portion of the curve, transfusion is considered to optimize oxygen delivery. However, this strategy may not be effective for patients in whom oxygen delivery is adequate.<sup>23</sup>

### Hemorrhagic Shock and Classification

Hemorrhage results in decreased cardiac output and total body ischemia, which cannot be corrected until circulating volume is restored. Acute loss of intravascular volume results in increased vascular tone and redistribution of blood flow to the heart and brain at the expense of cutaneous, splanchnic, and renal vascular beds. Acidosis develops as tissues switch from aerobic to anaerobic metabolism. This acidosis initially facilitates the unloading of oxygen at the tissues with a shift of the oxygen dissociation curve to the right. Urine output decreases as the kidneys work to retain water and sodium in response to decreased renal perfusion.

Without transfusion therapy, this correction requires the movement of fluid and protein from the interstitium to the plasma, or transcapillary plasma refill.<sup>24</sup> Initially triggered by a decrease in capillary hydrostatic pressure, this results in movement of protein-free fluid from the interstitium to the plasma. A second phase involves movement of protein into the plasma space in support of plasma oncotic pressure. This results in restoration of plasma volume and protein concentration with reduced oxygen-carrying capacity resulting from loss of red cell mass

by hemorrhage—that is, normovolemic anemia. Transcapillary refill is capable of sustaining a relatively fixed level of plasma volume, equal to approximately two-thirds of the initial plasma volume, irrespective of the rate of bleeding. Laboratory studies have shown that plasma refill reaches 33% by 0.5 hour after hemorrhage, and 50% by 3 hours, allowing fairly rapid restoration of circulating blood volume.<sup>25,26</sup> The amount of transcapillary refill is a function of the severity of pressure-driven hemorrhage but does not correlate with arterial pressure or cardiac output.

In evaluating injured patients, it rapidly becomes apparent that trauma patients are an extremely heterogeneous population. Each patient's resuscitative needs are different but can be broadly anticipated on the basis of initial hemodynamic status and the response to resuscitative efforts. The American College of Surgeons Committee on Trauma,<sup>27</sup> in an effort to guide and standardize the initial resuscitation of trauma patients, has defined four classes of shock (Table 39-1). Whereas crystalloid resuscitation alone is adequate for Class I and II shock, early consideration should be given to administering blood to patients in Class III and IV shock.

### The Trauma Patient

There are two distinct clinical settings in the care of the trauma patient. The first is the prehospital setting and the second is the in-hospital setting. Treatment of hemorrhagic shock starts in the prehospital setting with control of external bleeding. Once the patient is in the trauma bay, in-hospital management begins and it is perhaps best for the purposes of this chapter to approach this setting in multiple phases. The first phase is the initial resuscitation followed by either an intraoperative phase to stop bleeding or to definitively treat injuries, or a phase of observation, as in the management of solid organ injury. The

“intraoperative” phase can also take place in the angiography suite where bleeding can be controlled, most often in the presence of retroperitoneal and pelvic bleeding from pelvic fractures. The final phase of the care of the trauma patient is the recovery phase. Each of these phases differs with respect to the triggers and indications for transfusion therapy depending on the types of injuries that are inflicted and the type of patient that has sustained the injuries.

### Initial Resuscitation Phase of the Trauma Patient

Initial evaluation of a trauma patient, or the primary survey (ABCDEs), requires an assessment of the airway (A), breathing and ventilation (B), circulation (including the control of external hemorrhage) (C), neurologic status or disability (D), and exposure (E) of the patient. The initial resuscitation fluid of choice after trauma is an isotonic crystalloid solution, usually lactated Ringer's solution, and is often initiated in the prehospital clinical setting. The American College of Surgeons Committee on Trauma Advanced Trauma<sup>27</sup> recommends insertion of two large-bore intravenous catheters and immediate infusion of 2000 mL warmed crystalloid solution (or 20 mL/kg for a child). Because shock after trauma most often is hypovolemic, the response to initial fluid resuscitation determines subsequent resuscitative measures. The risk of hypothermia must be recognized and prevented during the resuscitation phase.

The response to fluid resuscitation determines which, if any, subsequent resuscitative measures are needed. A patient with minimal injury and normal vital signs with initial crystalloid resuscitation is called a *responder* to fluid resuscitation. The condition of these patients, by definition Class I or II hemorrhagic shock, remains hemodynamically normal without ongoing resuscitation. Such patients need evaluation and management of the injuries, but rarely need transfusion during the initial evaluation.

A patient in compensated shock, defined as hypovolemia coexistent with normotension but with serious metabolic derangement, often is difficult to identify. The conventional endpoints of resuscitation—heart rate, blood pressure, and urine output—are crude assessments of the adequacy of resuscitation. Early identification of compensated shock is facilitated by recognition of a transient response to fluid resuscitation. Patients in Class III shock often have hypotension and tachycardia, but vital signs improve or normalize in response to initial fluid resuscitation. During observation, such patients have physiologic signs of shock and need aggressive ongoing resuscitation to maintain relatively normal vital signs. Early identification of the site of hemorrhage and prompt surgical intervention minimize the period of hypoperfusion and the subsequent risk of multiple organ dysfunction. Transfusion with crossmatched or type-specific blood is indicated.

A patient in Class IV, or uncompensated, shock has pallor, diaphoresis, and apathy—clinical signs and symptoms that must be correctly interpreted. These patients also have tachycardia, hypotension, and tachypnea not responsive to aggressive fluid resuscitation with crystalloid and blood transfusion. Any injured

or burned patient who is cool and tachycardic is in shock until proven otherwise. These patients are described as *nonresponders*, in that their vital signs do not improve with aggressive resuscitative techniques. In this population, early identification of the site of hemorrhage and surgical intervention are paramount to survival. Early transfusion with group O RBCs is indicated.

The decision regarding early administration of blood, that is, in the trauma department, should be reserved for patients who have a transient response to initial resuscitation or those who have no response to resuscitation. These two groups have lost more than 30% of their blood volume or are still actively bleeding. Laboratory values of hemoglobin or hematocrit do not typically play a role in the determination of the need for blood transfusion in this patient population, but rather the clinical signs and symptoms of hemorrhage and response to resuscitation are the main determinants of the patient's estimated blood loss. Transfusion of blood in the trauma department must not delay definitive surgical intervention, and these patients should be transported to the operating room urgently.

In a retrospective study with 1000 trauma patients, Knottenbelt<sup>28</sup> found an interesting correlation between initial low hemoglobin level and mortality. He found that patients who came to medical attention in shock and died had significantly lower hemoglobin levels than did patients who did not arrive for treatment in shock. The mean hemoglobin levels of patients who died of hemorrhagic causes were significantly lower regardless of initial blood pressure. In this study, 48% of patients received no prehospital resuscitation, indicating that hemodilution was not the cause of a low initial hemoglobin level. Knottenbelt hypothesized that patients with a low hemoglobin level may have suffered blood loss exceeding their compensatory mechanisms. He concluded that a low initial hemoglobin level indicated severe, ongoing blood loss.

Once the decision to administer RBC transfusion has been reached, there are several options for the type of blood used. Blood ideally should be typed and crossmatched before transfusion, although this can take as long as 1 hour in many blood banks. This is an unacceptably long delay for a patient in Class III or IV shock. An alternative is transfusion of type-specific blood. Transfusion of type-specific blood is associated with a processing delay of 20 to 30 minutes, which in some circumstances still may be an unacceptable delay. In most trauma units, group O RBC units are immediately available and the most rapidly available choice in the care of trauma patients in hemodynamically unstable condition not responding to crystalloid resuscitation. Numerous studies have shown that administration of group O RBC units is a safe and rapid means by which to restore circulating red cell volume in a patient in severe shock.

It is of paramount importance to assess the adequacy of resuscitation; however, the search continues for valid markers. Many believe that base deficit accurately reflects the hemodynamic and tissue perfusion changes associated with shock and resuscitation.<sup>29</sup> Serum lactate is a reliable marker of hypoperfusion in hemorrhagic shock, because failure to clear lactate within 24 hours of injury has been associated with increased mortality.<sup>30</sup> Although serum markers may be helpful for guiding

the treatment of trauma patients, they are at best only a global measure of perfusion. Organ-specific monitoring, therefore, may be useful in ensuring adequate end-organ perfusion. Ivatury et al<sup>31</sup> have proposed gastric tonometry for such monitoring. They documented improved survival among patients whose gastric pH normalized within 24 hours. Other methods of measuring endpoints of resuscitation in the trauma patient include sublingual pCO<sub>2</sub> monitoring, tissue oxygen and carbon dioxide electrodes, and near infrared spectroscopy (NIRS) for the measurement of skeletal muscle oxyhemoglobin levels. Baron et al demonstrated in trauma patients with torso injuries that sublingual capnometry was able to differentiate between patients with varying amounts of blood loss<sup>32</sup> and was able to predict survival in hypotensive trauma patients equivalent to lactic acid levels and base deficit.<sup>33</sup> Measurement of skeletal muscle tissue oxygenation by two probes placed within the deltoid muscle include the polarographic tissue oxygen monitor (Licor, Integra, Plainsboro, NJ) and the NIRS (InSpectra, Hutchinson Technology, Hutchinson, MN). Each of these have been studied in the trauma patient.<sup>34</sup> They have not yet found a role in guiding resuscitation, but are predictive of postinjury complications. NIRS measures skeletal muscle oxyhemoglobin levels. It has been shown to be predictive of multiple organ failure in the trauma patient and has the added advantage that it is noninvasive.<sup>35</sup> Although these devices have the potential to help guide resuscitation, currently they are only adjuncts to other endpoints of resuscitation in the trauma patient and no particular one has been adopted widely.

## Intraoperative Phase of the Trauma Patient

### General Issues

The initial surgical treatment of a trauma patient involves rapid control of bleeding. In the case of severe abdominal trauma, this may necessitate use of “damage control” techniques or rapid packing of the abdomen to achieve hemostasis and rapid control of injuries to hollow viscera.<sup>36</sup> “Damage control” allows the quick, sometimes temporary control of bleeding so the anesthesia team can administer intravenous fluids and rapidly restore intravascular volume. The use of heated ventilator circuits prevents heat and moisture loss, but does not provide heat gain. The amount of heat lost from a normal person at room temperature is negligible, but it does become important in environments where inspired air is low temperature, such as the prehospital setting in cold climates.<sup>37</sup> Warmed intravenous fluids reduce the risk of hypothermia by preventing ongoing heat loss and provide some heat gain.<sup>38</sup> Ten liters of warmed intravenous fluids infused to a 70-kg male will increase the body temperature about 1.4°C. This contributes to the prevention of subsequent coagulopathy and acidosis associated with reduced body temperature and the infusion of large volumes of crystalloid and blood, which may be much cooler than body temperature. The use of “damage control” operative techniques in the care of the trauma patient is necessary when these physiologic factors are present.

The decision to perform intraoperative transfusion can be somewhat complex and involves both the surgeon and the

anesthesiologist. Despite a traditional hemoglobin-based transfusion trigger of 10 g/dL, numerous studies have documented the safety of a lower hemoglobin level.<sup>39,40</sup> In a study of Jehovah's Witness patients with anemia undergoing elective surgery, Spence et al<sup>39</sup> found that active bleeding was an independent predictor of survival only when hemoglobin level decreased to less than 4 g/dL. Low hemoglobin levels became an independent predictor of mortality only when they decreased to less than 3 g/dL. The application of these data to the care of a trauma patient must be tempered with the recognition that hemoglobin levels obtained during active hemorrhage may be falsely elevated because the intravascular and interstitial spaces have not equilibrated. In addition, the compensatory mechanisms of chronic anemia require more time to develop than that afforded by acute hemorrhage. Although results of these studies support the concept that the use of an arbitrary hemoglobin transfusion trigger is probably inappropriate, the intraoperative decision to administer transfusion must be based on an assessment of the patient's overall physiologic condition and the severity of ongoing blood loss.

Autotransfusion of the patient's blood has become a widely accepted practice in elective surgery and can be used as an adjunct to transfusion in the care of trauma patients. The benefits of this practice include the conservation of the blood supply and avoidance of complications related to allogeneic transfusion. Although intraoperative blood recovery and reinfusion has been successfully used in cases of thoracic trauma, its use in patients with abdominal injuries has not been universally applicable because of the risk of contamination from injuries to hollow viscera. The blood is typically anticoagulated with citrate or heparin, washed with normal saline, and then reinfused. Systems relying on filtration, rather than washing, were associated with disseminated intravascular coagulation and other complications and are generally no longer in use, with the possible exception of the reinfusion of mediastinal drainage fluid, which is largely defibrinated, following cardiac surgery.

### Solid Organ Injury

At the beginning of the 1900s, the mortality rate for nonoperative management of splenic injuries was 100%; thus, splenectomy was readily accepted as the standard of care for splenic rupture. However, King and Schumaker reported on overwhelming postsplenectomy sepsis among infants who had undergone splenectomy.<sup>41</sup> Despite this report, the practice of splenectomy remained unchallenged through the first half of the 20th century, until pediatric surgeons began to realize that children in hemodynamically stable condition with splenic injuries could be safely observed. With the advent of improved imaging techniques, including computed tomography (CT) of the abdomen and pelvis, splenic injuries can be identified in patients with normal hemodynamic values. The pediatric experience has been extended to the treatment of adults who have sustained trauma but who are in hemodynamically stable condition. Benefits of splenic preservation include avoidance of overwhelming post-splenectomy sepsis and avoidance of unnecessary laparotomy.



In the 1990s, these principles were extended to the nonoperative treatment of selected patients with liver trauma.<sup>42</sup>

In determining whether a patient needs operative therapy for a splenic injury, the most important factor is hemodynamic stability. Patients in hemodynamically unstable condition (approximately 35% of adult patients with splenic injury and 3% in children<sup>42</sup>) need exploration and splenectomy. Splenorrhaphy is an option, but with the nonoperative management of many splenic injuries, it is not utilized in the adult trauma patient with failed nonoperative management. Generally, surgery is indicated in the worst of the splenic injuries that have failed nonoperative management and require removal. Splenorrhaphy is generally reserved for preservation of the spleen when it is an incidental finding upon laparotomy and not a source of major hemorrhage, especially in the immunologically immature pediatric patient.<sup>43,44</sup> However, 65% of patients with splenic injury arrive in hemodynamically stable condition and are candidates for nonoperative treatment. Nonoperative management fails when hemodynamic instability necessitates exploration, or hemoglobin level decreases, necessitating transfusion, but there is some variation between practitioners regarding the criteria for operative intervention. Most patients with splenic injury do not need transfusion. However, children under the age of 5 have a higher risk of postsplenectomy sepsis. The relative risk of a 1- or 2-unit transfusion (if the patient is in otherwise stable condition) seems a reasonable alternative to splenectomy.<sup>45</sup> Adjuncts to improve the success of nonoperative management of splenic injuries include the use of angiography. Schurr et al<sup>46</sup> described a contrast blush on helical CT scans of the spleen. This finding corresponded to a traumatic pseudoaneurysm on an angiogram and can be successfully treated in the hemodynamically stable patient.

In 1990, Knudson et al<sup>47</sup> described 52 patients with blunt liver trauma who were observed carefully with serial CT. No failures of nonoperative management were reported. This was followed by a report<sup>48</sup> from the group at the University of Tennessee at Memphis, documenting the safety of nonoperative management of even the most severe liver injuries. The authors found transfusion requirements were markedly lower than for patients with severe liver injury who underwent surgery. The only criterion for initial nonoperative management was hemodynamic stability. As with splenic trauma, early identification of contrast extravasation on helical CT scans of the liver with subsequent angiographic embolization of the bleeding vessel has improved the success rate of nonoperative management.<sup>49</sup>

### Recovery Phase of the Trauma Patient

The time between operative or nonoperative treatment of a patient who has sustained trauma until discharge from acute care may be the period in which transfusion can be decreased the most without compromising the patient's condition. During this period, there is no ongoing blood loss, so dramatic changes in hematocrit are not expected. The decision to administer RBC transfusion during this period should be based not on

maintaining an arbitrary hematocrit but on results of assessment of the physiologic need for transfusion.

Traditional teaching that all patients should have a hematocrit of 30% (or a hemoglobin level of 10 g/dL) for optimal oxygen delivery has not been borne out in recent studies. Persons with normal blood volume and normal cardiopulmonary function tolerate hemoglobin levels as low as 5 g/dL.<sup>50</sup> A prospective, randomized controlled trial in the care of critically ill patients demonstrated patients younger than 55 years or patients with Acute Physiology and Chronic Health Evaluation II (APACHE II) scores less than 20, may have a reduced mortality if a restrictive transfusion strategy is adopted and hemoglobin levels are maintained at 7 to 9 g/dL.<sup>51</sup> The same reduction in mortality is not seen in a subgroup analysis of resuscitated critically ill trauma patients or brain-injured patients. In fact, those transfused to maintain the hemoglobin level of 7 to 9 g/dL vs 10 to 12 g/dL did not have any difference in outcomes.<sup>52,53</sup> However, further prospective trials should be performed to confirm these findings, especially in a high-risk population such as brain-injured patients. Moderate to severely brain-injured patients may warrant earlier operative management of solid organ injury, especially stable patients with splenic injuries, because of the association of hypotension with increased mortality. If the mortality from the brain injury is worse with hypotension, nonoperative management of moderate splenic injuries in the stable patient may not be worth the risk of failure and exposure to hypotension. Hypothermia is associated with mortality in patients with brain injury and is also a result of severe hemorrhage and transfusion in the trauma patient.<sup>54</sup> Decisions to administer RBC transfusion should be based on evidence of symptomatic anemia, including chest pain, dyspnea, fatigue, and significant tachycardia. In the absence of symptoms, RBC transfusion is not warranted. A more liberal transfusion trigger is probably warranted in the care of those with underlying severe cardiovascular disease.

### Complications of Massive Transfusion Therapy

Massive transfusion has been defined as replacement of a patient's entire blood volume in 24 hours, transfusion of more than 10 RBC units, or replacement of more than 50% of the circulating blood volume within 3 hours.<sup>55,56</sup> Massive resuscitation with fluids and blood components after hemorrhagic shock is associated with complex metabolic derangements and high morbidity and mortality rates. The clinical hallmark of severe hemorrhagic shock is the lethal triad of acidosis, hypothermia, and coagulopathy (Fig 39-2).

Banked blood undergoes a number of metabolic and structural changes.<sup>56</sup> When it is transfused in large volumes, the blood can theoretically cause severe derangements in physiologic values. It appears, however, that the severity and duration of shock rather than the volume of transfused blood are the primary determinants of physiologic derangement and ultimately patient outcome.<sup>57,58</sup> It is vital that patients receiving massive transfusion also receive adequate fluid resuscitation such that oxygen delivery and organ perfusion are maintained. Survival after

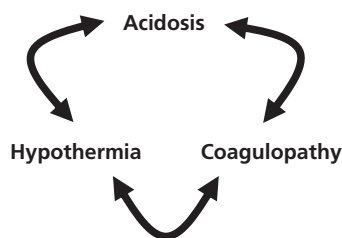


**Figure 39-2.** The lethal triad.

massive transfusion is no longer uncommon. One patient received 186 units of blood and blood components within 12 hours and had a successful outcome.<sup>59,60</sup>

One problem associated with massive resuscitation is the occurrence of microvascular bleeding, or so-called medical bleeding. This is to be clearly differentiated from surgical bleeding. Medical bleeding is associated with mucous membrane bleeding, oozing from surgical wounds and raw tissue surfaces, oozing from catheter sites after application of direct pressure, and generalized petechiae and enlarging ecchymoses.<sup>61</sup> It has been recommended that prophylactic transfusion of platelets and Fresh Frozen Plasma (FFP) be administered according to the volume of RBCs transfused.<sup>61-63</sup> However, this practice pattern may not decrease the risk of microvascular bleeding and may markedly increase the number of donor units transfused.

Several studies have evaluated changes in levels of coagulation factors and platelet count and their role in microvascular bleeding after trauma and massive transfusion.<sup>62-64</sup> These studies document a decrease in both coagulation factors and platelet counts. One explanation is the development of a washout phenomenon, or dilutional coagulopathy, resulting from the use of stored blood deficient in both platelets and coagulation factors.<sup>64</sup> Although dilution appears to play a role in thrombocytopenia and coagulopathy after massive resuscitation, it does not account for all changes after massive transfusion.

In a regression analysis of thrombocytopenia after massive transfusion, Reed et al<sup>61</sup> found that only 35% of the decrease in platelet count could be attributed to dilution. They found further that 50% of patients with microvascular bleeding needed large volumes of platelet transfusions well after the period of massive resuscitation. This finding suggested that factors in addition to dilution must play a role in persistent thrombocytopenia in these patients. In a related study,<sup>64</sup> investigators hypothesized that platelet consumption accounted for this persistent thrombocytopenia, which was related to the pathophysiologic response accompanying lung injury, brain injury, massive tissue injury, sepsis, and endothelial damage.

Several authors have examined the concentration of coagulation factors to determine whether dilution plays a contributing role. Martin et al<sup>65</sup> found that although levels of coagulation factors decreased significantly at the end of a shock insult, the levels remained much higher than those considered adequate for normal hemostasis. Reed et al<sup>61,62</sup> evaluated coagulation studies

in the treatment of patients who received massive transfusions. They found mild to moderate prolongation of prothrombin time (PT) and partial thromboplastin time (PTT) were common in this patient population but were not predictive of microvascular bleeding. Regression analysis showed that 15% to 35% of the elevation of the PT and PTT could not be attributed to coagulation factor deficiencies. This finding indicated that other, unidentifiable factors had a role in the elevation of the PT and PTT.

Although no one study alone can confirm which patients at risk of microvascular bleeding will benefit from transfusions of platelets and FFP, certain tests are more useful than others. A PT less than 1.3 times control value has a 94% predictive value in indicating the absence of microvascular bleeding. Although the sensitivity of PT or PTT in identifying microvascular bleeding is only 50%, values greater than 1.8 times control value are 96% specific for microvascular bleeding. Bleeding time is of no value in determining which patients will have microvascular bleeding. The most sensitive laboratory predictors are a platelet count less than 50,000/ $\mu$ L and a fibrinogen level less than 0.5 g/L, with a combined sensitivity of 87% and a negative predictive value of 96%. This has led to the following recommendations: platelet transfusion for patients with a platelet count less than 100,000/ $\mu$ L and evidence of microvascular bleeding; prophylactic platelet transfusion for patients with a platelet count less than 50,000/ $\mu$ L; and administration of supplemental FFP or cryoprecipitate to patients with fibrinogen levels of 0.8 g/L or less.<sup>62</sup>

Hypothermia is a contributing factor to platelet and coagulation factor dysfunction after massive transfusion. Villalobos et al<sup>66</sup> studied the effects of hypothermia on platelets in dogs and found that thrombocytopenia was caused by sequestration of platelets by the liver, spleen, and other sites in the portal circulation rather than by platelet destruction. No change in platelet production was found during either cooling or rewarming.

Reed et al<sup>67-69</sup> reported on coagulation factor dysfunction secondary to hypothermia. Standard coagulation testing in the laboratory typically is performed at 37°C. This method detects clotting abnormalities only if the actual factor content is decreased. The function of the coagulation cascade, however, depends on enzymatic activity, which can be adversely affected by hypothermia. The result is prolongation of clotting times even in the face of normal factor concentrations. Investigators<sup>68</sup> found that the PTT was prolonged at all temperatures less than 35°C and that the PT was prolonged at temperatures less than 33°C. They concluded that if clinical oozing exists in a patient with hypothermia and results of coagulation studies performed at 37°C are within the normal range, rewarming the patient is more appropriate and more effective than empiric transfusion of FFP.

Recognition of the contributions of hypothermia to the development of medical bleeding has revolutionized the field of trauma surgery. The concept of the “damage control” laparotomy has been reborn. In this procedure, all efforts are directed at control of surgical bleeding and containment of intra-abdominal contamination. However, the operation is truncated to minimize the “on-table” time. The patient often is closed with laparotomy packs in place

in a temporary abdominal wall closure.<sup>34</sup> The patient is returned to the surgical intensive care unit, where aggressive rewarming and resuscitation continue. Only when acidosis, coagulopathy, and hypothermia have been corrected is the patient returned to the operating room for definitive management of injuries.

It is suggested that storage of blood longer than 14 days is related to the side effects, and in many trauma centers with a large demand for blood components, many of the older units of blood in circulation are sent to high-volume centers where they will more likely be used before their expiration. In other words, trauma patients in busy trauma and medical centers tend to receive older blood. Transfused leukocytes have been implicated as the cause of many transfusion reactions, but older blood also has red cell morphology changes, adenosine triphosphate depletion, 2,3-diphosphoglycerate depletion, and other biochemical changes (see Chapter 4). Use of prestorage leukocyte-reduced blood components should in theory reduce the immunomodulatory side effects of blood transfusion, but there are many other contributing factors that alter blood in storage.<sup>70</sup> It has been demonstrated that trauma patients with multiple organ failure who were transfused in the first 12 hours of admission received more blood that was greater than 14 days old.<sup>71</sup> In addition, blood greater than 14 days old was an independent predictor of serious infection.<sup>72</sup>

Currently, the use of whole blood is limited to the military and war-related trauma. Although it is possible to move refrigerators and freezers close to areas of combat, storing platelets at the proper temperature and working within the expiration date are challenging. Thus, the military uses whole blood transfusion or their “walking donor” program. Whole blood transfusion is utilized as a lifesaving therapy in combat with otherwise untreatable coagulopathy in the absence of appropriate blood components.<sup>73</sup> Whole blood is not used in the civilian trauma center. A retrospective review of patients who received whole blood transfusion from January 2001 to December 2004 revealed that of the 5294 transfusions given to 3287 patients, 545 units of whole blood were transfused to 87 (3%) patients.<sup>74</sup> The United States military advocates the use of whole blood transfusion only for victims with life-threatening injuries and hemorrhage.

## The Burn Patient

### Initial Resuscitation of the Burn Patient

The large number of resuscitation formulas available for use in the treatment of thermally injured patients is a tribute to the fact that no one formula is an accurate predictor of the fluid requirements of every patient. No formula replaces the role of the physician, who must continually assess the adequacy of resuscitation. Ongoing controversies about resuscitation involve the use of colloid, the differences between adults and children, and the influence of inhalation injury on fluid requirements.

In the first 24 hours of resuscitation, most patients need 2 to 4 mL/kg of fluid per percentage of total body surface area (BSA) burned. This fluid is given as lactated Ringer’s solution. The first

half of the calculated requirement is given over the first 8 hours, measured from time of injury, not from the beginning of resuscitation. The rest is given over the next 16 hours (Parkland formula).<sup>75,76</sup> Children also need maintenance fluids containing dextrose in addition to the resuscitative fluid. The presence of inhalation injury increases the volume of fluid needed to achieve adequate resuscitation. Whether colloid is used in the first 24 hours is determined on a case-by-case basis; administration of colloid should not be routine, but is utilized in the severely burned patient who is not responsive to resuscitation. Regardless of the volume of resuscitative fluid initially chosen, ongoing resuscitation should be dictated by the patient’s response to resuscitation. Urinary output of 1 mL/kg/hour is an adequate measure of resuscitation.

Release of vasoactive mediators from injured tissue results in a diffuse capillary leak starting soon after injury. This loss of microvascular integrity results in extravasation of crystalloid and colloid solutions for the first 18 to 24 hours after thermal injury. This pathophysiologic process explains the enormous fluid requirements of burn patients for the first 24 hours. This is the reason that most burn resuscitation formulas incorporate the use of colloids after 24 hours—microvascular integrity apparently is restored at that time. After 24 hours, colloid remains largely intravascular. Colloid, generally 5% albumin, is infused at a dose based on burn size (generally 0.3 to 0.5 mL/kg per percentage of total BSA burned over 24 hours). During this period, crystalloid requirements decrease markedly.

### Transfusion Therapy in the Care of Thermally Injured Patients

As with severely injured trauma patients, thermally injured patients often have anemia throughout the hospital course. The cause of this anemia is very different from that of the anemia of trauma.<sup>77-81</sup> The factors that predominate as the cause of anemia vary with respect to time from injury; they must be considered in evaluation for transfusion.<sup>78,79</sup>

Soon after burn injury, anemia is caused by direct destruction of erythrocytes within the cutaneous circulation and by hemorrhage into the burn wound. As time progresses, the factors resulting in anemia include hemolysis of injured erythrocytes and blood loss during dressing changes. Further hemolysis also occurs as blood flows through injured tissue and burn eschar. Additional blood loss occurs during burn wound débridement, split-thickness skin grafting, and postoperative dressing changes. Because of the stress associated with thermal injury, blood loss can occur in the gastrointestinal tract, as well as from unnecessary “routine” blood work.

The response of burn patients to anemia is different from that of trauma patients.<sup>78-81</sup> Several, but not all, studies have documented an increase in erythropoietin level after thermal injury as well as a paradoxical decrease in reticulocyte numbers.<sup>80,81</sup> In a study with 27 patients, erythropoietin levels were inversely related to hemoglobin levels, but patients had persistent reticulocytopenia.<sup>81</sup> The results of this study suggested a relative resistance

to erythropoietin at the marrow level. An elevated level of circulating erythropoietin did not result in an expected increase in erythrocyte production. It appears that the erythropoietic response to thermal injury is inversely proportional to the size of the burn.<sup>80</sup> Results of studies of the use of recombinant erythropoietin in the management of postburn anemia have not been encouraging. A study with pediatric burn patients did not show a statistically significant increase in hematocrit for either burn patients or healthy volunteers, although increases in reticulocyte count occurred.<sup>77</sup> With no effect in either group, it is questionable if the dose or schedule of the drug was adequate. Similar studies in adult patients with 25% to 65% total BSA burns did not show a significant difference in reticulocyte counts, transfusion requirements, hemoglobin, hematocrit, or iron binding. There was, however, a significant increase in reticulocyte counts in patients with burns from 25% to 35%.<sup>82</sup> More studies with perhaps higher doses need to be performed to determine if erythropoietin is effective in this population at different doses or administration regimens, or if the benefit is not in the increase in the hemoglobin, but other properties of erythropoietin.

The relative resistance of the marrow of thermally injured patients to erythropoietin may be caused by an inhibitory factor in the serum. A study was performed in which serum from patients with more than 20% total BSA burned was cultured with mouse marrow. The authors found the number of erythroid colonies was significantly less in the presence of sera from burn patients even when erythropoietin was added to the system. When erythropoietin activity was assayed *in vivo*, no decrease in activity was detected. This finding suggests the inhibition did not occur at the level of the erythropoietin.<sup>79</sup> Another study showed that erythroid colony-forming cells appeared to return to normal as healing occurred. This study also showed that granulocytopoiesis and thrombocytopoiesis were unaffected and proceeded at an accelerated rate.<sup>78</sup> These authors postulated that redirection of the pluripotent stem cells away from the erythroid line to the granulocyte and monocyte cell lines may offer a survival advantage to burned patients because infection and sepsis are major sources of morbidity and mortality in this population.

As in the care of trauma patients, the safety of lower hemoglobin and hematocrit levels in the care of burn patients has been demonstrated.<sup>83,84</sup> Mann et al<sup>84</sup> retrospectively examined the transfusion requirements of patients with burns of more than 10% total BSA who needed at least one operation. They found a fivefold decrease from 1980 to 1990 in the volume of blood transfused per percentage total BSA without adverse cardiac complications or increased morbidity. Because of their findings, the authors recommended that healthy patients do not need transfusion unless the hematocrit decreases to less than 15% to 20% in patients who need one operation, or 25% in patients who need more than one operative procedure. As with trauma patients, thermally injured patients with underlying cardiovascular disease should be treated with a more liberalized transfusion policy. Sittig and Deitch<sup>83</sup> confirmed the foregoing findings and found a greater than a threefold decrease in transfusion

requirements when a more selective transfusion policy was implemented whereby 86% of blood transfused was given at the time of an operative procedure.

Intraoperative use of hemostatic agents can further decrease transfusion requirements. It is well known that intraoperative blood loss is the limiting factor of the extent of excision and grafting performed. Thrombin spray and laparotomy pads saturated with thrombin and epinephrine have been used extensively to limit intraoperative blood loss.<sup>83,85</sup> The use of tourniquets for excision and grafting of extremities has further decreased blood loss.<sup>86</sup> Fibrin sealants may prove effective. Results of preliminary studies of the use of intraoperative blood recovery techniques show recovery and reinfusion of approximately 40% of shed blood without adverse inflammatory or infectious complications.<sup>87</sup> As in the care of trauma patients, the importance of normothermia in preventing coagulopathy is recognized, and patients are aggressively warmed at the time of surgery.

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## Adjuncts in Transfusion Therapy for Trauma and Burn Patients

Although crystalloid and blood are the standards in initial resuscitation of trauma patients, there is active investigational interest in developing alternative resuscitative materials. While it is clear that blood is an effective resuscitative fluid that rapidly expands circulatory volume and improves oxygen transportation, it has many disadvantages. It must be crossmatched; it has a short shelf life; it must be stored under closely monitored conditions; it is associated with a variety of immunologic and infectious complications; and its supply relies on the altruism of the donor population.

### Blood Substitutes (Oxygen Carriers)

There have been numerous attempts at developing synthetic products that will aid in oxygen-carrying capacity, mostly based on the use of solutions of hemoglobin-based oxygen carriers or perfluorocarbon emulsions. All of them offer the advantages of long shelf life, permissive storage conditions, absence of the need for compatibility testing, and virtual absence of infectious risks. Most oxygen carriers under development are based on either human or animal hemoglobin. The source of human hemoglobin is outdated RBCs. The disadvantage of human hemoglobin relates to a limitation in the availability of outdated RBCs; less than 3% of the 15.1 million units of blood donated in the United States are being discarded. Animal hemoglobin, particularly bovine hemoglobin, is potentially inexpensive and is readily available, although uncertainty regarding the purification of animal pathogens remains a concern as does the antigenicity of the components.<sup>88,89</sup>

Several companies are conducting efficacy trials of hemoglobin-based oxygen carriers. One company, Baxter Healthcare (Deerfield, IL) terminated work with a diaspirin cross-linked hemoglobin when multicenter trials with trauma patients with hypotension and stroke patients showed increased

multiple organ failure, acute respiratory distress syndrome, and mortality during interim review.<sup>90</sup> PolyHeme (Northfield Laboratories, Evanston, IL) has completed two Phase III trials and appears to lack the marked vasopressive effects of diaspirin cross-linked hemoglobin. A randomized, Phase II clinical trial of PolyHeme vs RBCs in the treatment of trauma patients showed a lower overall RBC transfusion requirement; there was no difference in circulating hemoglobin level, and there were no adverse events.<sup>91</sup> Comparison of patients who received PolyHeme during three different studies, and a historical control group composed of consecutive surgical patients who declined blood transfusion for religious reasons, showed a mortality rate of 25% in the PolyHeme group vs 65% in the group that did not receive the oxygen carrier.<sup>92</sup> A Phase III trial using PolyHeme as a resuscitative component in prehospital trauma patients has been conducted but the data have not yet been published. A bovine hemoglobin-based solution, Hemopure (Biopure Corporation, Cambridge MA), appears to have some vasopressor effects. One Phase III trial has been completed; plans for another Phase III trial are under discussion with the Food and Drug Administration. Hemopure has been licensed for use in clinical practice in South Africa since 2001. Sangart (San Diego, CA) has developed a PEGylated human hemoglobin product with minimal vasoactivity, which has entered Phase II clinical trials in Europe and the United States.<sup>93</sup>

Perfluorocarbons are synthetic molecules with high solubility for oxygen and carbon dioxide. Compared to blood however, their oxygen-carrying capacity is quite low, so patients must receive supplemental oxygen. The compounds are not derived from other animal products and, therefore, do not have the antigenic properties that accompany bioproducts. However, they can cause activation of complement, malaise, and flu-like symptoms; other side effects include thrombocytopenia, splenomegaly, and hepatomegaly. The perfluorocarbon emulsions are cleared by the reticulo-endothelial system relatively quickly with a half life of 12 to 24 hours. Currently, there are no perfluorocarbons in clinical trials mostly because of adverse effects found early in clinical trials and the lack of efficacy.<sup>89</sup>

## Colloid and Crystalloid Solutions

Although blood substitutes remain in clinical trials, other solutions have been evaluated for use in immediate resuscitation of trauma patients. It must be emphasized that colloid solutions are not effective in improving oxygen delivery in the hemorrhaging trauma patient. Colloid solutions are theoretically more efficient than crystalloid at restoring intravascular volume. The colloid-crystalloid debate is at least 50 years old. A potential indication for colloid administration is ongoing resuscitation of critically injured patients. The SAFE study<sup>94</sup> published in 2004 examined the choice of resuscitation with 4% albumin vs normal saline in intensive care units (burns were excluded) and found no difference in mortality at 28 days. The subgroup analysis of trauma patients was small, but mortality trended toward worsened mortality in the albumin group. Post-hoc analysis of the brain-injured

patients revealed higher mortality in patients who received albumin, especially in those with more severe brain injury.<sup>95</sup> There is little doubt that smaller volumes of colloid than of crystalloid are needed to restore intravascular volume and this is demonstrated in the studies described above. Meta-analysis from the Cochrane database has not found any mortality benefit to the use of colloids from the combined results of 63 eligible trials.<sup>96</sup>

To clarify the colloid vs crystalloid debate, several studies have examined outcomes, including survival and generation of extravascular lung water. Investigators in favor of crystalloid resuscitation argue that after severe trauma, there is depletion of interstitial volume as well as intravascular volume, a deficit not adequately addressed with colloid resuscitation. Crystalloid has been proposed as the best resuscitation fluid because it has been shown to restore the volume of both the intravascular and interstitial spaces without worsening pulmonary function.<sup>97</sup> Pulmonary lymphatic vessels increase their flow significantly in animals, and the increase likely occurs in humans, minimizing the development of pulmonary edema after crystalloid resuscitation. Proponents of colloid resuscitation, however, believe that resuscitation of the intravascular component alone is beneficial and that use of colloid may decrease the incidence of pulmonary edema.<sup>98</sup> Although this debate has been subjected to several meta-analyses, the results are conflicting because of highly heterogeneous patient populations and study designs.

Advocates of crystalloid use note the lower cost of crystalloids than of colloids, although larger volumes are needed to reach similar endpoints. Although colloids remain in the intravascular space, in states of increased vascular permeability, colloids can leak into the interstitium, increasing the colloid oncotic pressure of the extravascular space. This increased oncotic pressure can hamper mobilization of third-space fluids after the acute resuscitative phase has been completed. Velanovich<sup>99</sup> reviewed eight randomized, prospective trials comparing crystalloid to colloid resuscitation. He found a 5.7% relative decrease in mortality in the group receiving crystalloid resuscitation. When nontrauma trials were excluded, a 12.3% decrease in mortality favored crystalloid resuscitation.

## Hypertonic Saline Solution

Several studies have examined use of hypertonic saline solution in the resuscitation of animals and humans from shock. Hypertonic saline solution has the advantage of requiring smaller volumes to reach endpoints similar to those of standard crystalloid resuscitation. This makes hypertonic saline solution particularly attractive for use in the prehospital setting, but it does not aid in oxygen-carrying capacity. Administration of hypertonic saline solution has been shown to elevate mean arterial pressure and cardiac output and to increase renal, mesenteric, total splanchnic, and coronary blood flow. Hypertonic saline solution also causes a small and transient increase in circulatory volume by means of transcapillary refill. These effects are well established after controlled hemorrhage. However, the utility of hypertonic saline solution in the management of uncontrolled hemorrhage has yet to be firmly established.

Gross et al,<sup>100</sup> using a model of uncontrolled hemorrhage in rats, found increased blood loss and mortality after administration of hypertonic saline solution. They concluded that hypertonic saline resuscitation must be delayed until after definitive control of hemorrhage.<sup>100</sup> This study was criticized for its use of an anesthetic agent that may have exacerbated hypotension and affected the outcome. Coimbra et al<sup>101</sup> compared hypertonic saline and shed blood resuscitation with crystalloid and shed blood resuscitation in a model of hemorrhage and cecal ligation and puncture in rats. They found improved survival among animals resuscitated with hypertonic saline solution and shed blood, improvement in pulmonary histologic features, and decreased local peritoneal sepsis. They hypothesized that resuscitation with hypertonic saline solution caused less impairment in cellular immune function, possibly contributing to the improvement in survival that they documented.

In a prospective, randomized trial with 105 patients in hypovolemic shock, Younes et al<sup>102</sup> found that infusion of 250 mL of hypertonic saline solution was not associated with any complications and did not affect mortality. It did improve mean arterial blood pressure significantly, acutely expanded plasma volume 24%, and significantly reduced the volume of crystalloid and blood needed for resuscitation. A multicenter trial evaluated the use of hypertonic saline and dextran in the prehospital treatment of trauma patients with hypotension.<sup>103</sup> The investigators found improved survival among patients undergoing surgery who received 7.5% sodium chloride in 6% dextran 70 compared to those receiving the same volume of normal crystalloid solution. The difference reached statistical significance, although there was no overall improvement in survival rate. Use of hypertonic saline solution and dextran also appeared to be associated with a lower incidence of acute respiratory distress syndrome, renal failure, and coagulopathy. Reports by others have described a similar survival benefit among patients receiving hypertonic saline solution vs conventional resuscitative fluids in prehospital therapy for hypotension associated with trauma.<sup>104</sup> Two studies have looked at the immunomodulating effects of hypertonic saline (7.5%)/dextran (6%) in the resuscitation of trauma patients in hemorrhagic shock. These studies demonstrated the anti-inflammatory modulation of cytokines and cellular components that occur with the administration of this solution compared to normal crystalloid solutions (lactated Ringer's and 0.9% sodium chloride).<sup>105,106</sup>

### Recombinant Human Erythropoietin

Recombinant human erythropoietin (rHuEPO) does not have a role in the actively bleeding trauma patient, but has been studied in the treatment of critically ill patients with a prospective, randomized control study.<sup>107</sup> This study demonstrated an overall reduction in RBC transfusions for the patients receiving rHuEPO 40,000U weekly for three doses vs placebo. This effect was not observed until about 1 week following the administration of rHuEPO. Post-hoc subgroup analysis looked at trauma patients; nontrauma surgical patients; and nontrauma, nonsurgical medical patients. There was a trend in the trauma patients

receiving rHuEPO to have a lower mortality (4.8% receiving rHuEPO vs 10.4% receiving placebo), but this was not statistically significant.<sup>107</sup> The cost of rHuEPO balanced against the benefit of its use (avoidance of transfusion-related complications) does not justify its use in the critically ill patient if there is no mortality difference.<sup>108</sup> Corwin et al<sup>109</sup> repeated this study of rHuEPO in critically ill patients in 2007. The repeat study addressed the trend toward a mortality difference seen in the first study published in 2002. The results showed a difference in 29-day mortality in critically ill patients receiving rHuEPO (8.5% vs 11.4%,  $p = 0.02$ ), and specifically in trauma patients admitted to the intensive care unit for more than 48 hours (3.5% vs 6.6%,  $p = 0.04$ ). This difference in mortality was maintained at 140 days, but not seen in medical patients or nontrauma surgical patients. Unlike the previous study, there was no difference in the number of transfusions given between the treatment and placebo groups, which may be a reflection of the adoption of restrictive transfusion strategies in critically ill patients. In addition, post-hoc analysis showed there was a significant increase (20.3% vs 12.8%,  $p = 0.008$ ) in the treatment group for thromboembolic events. This increase in events was not demonstrated with patients who received early administration of heparin.<sup>109</sup>

Independent of the effects of rHuEPO on blood transfusion and hematocrit, there have been multiple animal studies demonstrating what are thought to be anti-inflammatory, neurogenic, and anti-apoptotic characteristics of the drug in brain-injured animal models. In many of these studies, prevention of post-injury brain atrophy, behavioral abnormalities, and cognitive dysfunction have been demonstrated.<sup>110,111</sup> What remains to be proven is the effect, if any, in the brain-injured patient in local and long-term outcomes.

### Recombinant Activated Factor VIII

Another agent that has been studied as an adjunct to transfusion in the trauma and burn patient is recombinant activated Factor VII (rFVIIa). This drug is licensed for the treatment of patients with Factor VII deficiency or Factor VIII inhibitors. It has been studied in hemorrhage associated with surgical procedures, trauma, and burns. The "lethal triad" of coagulopathy, hypothermia and acidosis is a well-known clinical scenario and resuscitation is often targeted to correct these variables. Administration of rFVIIa aims at correcting the coagulopathy limb and is known for being very effective in the nontrauma patient with bleeding disorders. For this reason, there is interest in finding a utility in the coagulopathic trauma patient.

A multicenter and multinational study was conducted to look at the effectiveness of rFVIIa in reducing the number of blood transfusions in severely injured blunt and penetrating trauma patients. There was a significant reduction in the number of transfusions required (reduction of 2.6 RBC units) and the need for massive transfusion was reduced from 33% to 14% (massive transfusion defined as >20 RBC units) in the severely injured blunt trauma population.<sup>112</sup> Adverse events, mortality, and other clinical endpoints were the same in each of the treatment groups;

however, there was a trend toward improvement in the incidence of acute respiratory distress syndrome in the blunt trauma treatment group and multiple organ failure in the penetrating trauma treatment group. The lack of effect on the number of RBC transfused in the penetrating trauma group could be related to the difference between surgical and medical bleeding. The administration to patients in combat with penetrating injuries appeared to decrease the number of transfusions by 20% in those requiring massive transfusion if it was received early (before transfusion of 8 RBC units) vs late (after 8 RBC units were given).<sup>113</sup>

Of concern with the use of rFVIIa is the risk for thromboembolic complications, especially when arterial injury is present. The incidence of thromboembolic complications with use of rFVIIa is from 3% to 9.4%<sup>112,114</sup> and can be a cause of severe morbidity and mortality. Dose-dependent or temporal relationships to thrombotic events have not yet been elucidated, but as this drug is used more as an adjunct to transfusion therapy in the severely injured patient, these details may be determined. Although the potential exists for therapeutic benefit, rFVIIa has not been studied in a prospective fashion in brain-injured trauma patients and there no recommendation for its use in this setting.

Use of rFVIIa has also been described in the burn patient. There is often major blood loss with the excision of burned skin and grafting. A single center pilot study of 18 patients<sup>115</sup> showed a significant decrease in the number of blood components transfused (0.9 vs 2.2,  $p = 0.0013$ ) and more specifically the number of RBC units transfused (0.5 vs 1.1,  $p = 0.004$ ) when rFVIIa (40 $\mu$ g/kg) was used at skin incision and redosed intraoperatively at 90 minutes. There was also a trend toward improved graft survival and reduction in multiple organ failure in the group receiving rFVIIa. This was, however, a pilot study and a study with better power should be conducted to confirm these results.

## Summary

Massive hemorrhage and its consequences of hypothermia, acidosis, and coagulopathy continue to be the leading cause of death during resuscitation of severely injured trauma patients. The early use of blood and blood components combined with aggressive crystalloid resuscitation and early surgical intervention has significantly decreased mortality. However, the risk of disease transmission has prompted reevaluation of the transfusion practices of both trauma and burn surgeons. Although it is recognized that these patients tolerate a lower hemoglobin level than was originally thought, the lower limit of safe hemoglobin level is not known. Although data from other surgical populations have helped to demonstrate that previously accepted transfusion triggers are not appropriate, the unique characteristics of injured patients do not allow universal application of these findings. It is possible to optimize transfusion practices once it is realized that injured patients experience distinct clinical phases based on time from injury. Although blood transfusion continues to be a lifesaving therapy, judicious use of blood and blood

components can provide maximal benefit and minimize unnecessary risk to the patient after injury.

## Disclaimer

The authors have disclosed no conflicts of interest.

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## 40

# Transfusion Therapy in Solid-Organ Transplantation

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Recent clinical progress in organ transplantation has been tempered by the limited supply of organs.<sup>1</sup> The blood bank has a vital place on the hospital-wide team that makes transplantation possible for severely ill patients. Approximately 1% of all Red Blood Cell (RBC) and platelet transfusions and approximately 5% of all plasma and cryoprecipitate transfusions are used in organ transplantation surgery.<sup>2</sup> Blood banks also play an increasing role in recent efforts to prevent ABO- and HLA-mediated graft rejection.

In the United States, 257 programs perform organ transplantation. Table 40-1 shows the number of transplants of each organ performed in the United States in 2006.<sup>3</sup> Also shown are the 3-year graft and patient survival percentages for transplantation procedures performed on adults in the United States in 2001 with follow-up data through 2004. Pediatric results are similar.

## Organ Procurement

In the United States, organ procurement and allocation are regulated by the Health Resources and Services Administration (HRSA) of the Department of Health and Human Services. Under the authority of the National Organ Transplant Act [Title 42 US Code (USC), Section 273-274], HRSA oversees the Organ Procurement Transplant Network (OPTN) [Title 42 Code of Federal Regulations (CFR), Section 121].<sup>4,5</sup> The law for Medicare participation (42 USC 1320b-8) requires membership in the OPTN for all transplant centers (42 CFR 482.45) and organ procurement organizations (42 CFR 486.301-348). The OPTN in turn formulates policies required for its members.<sup>6</sup> The OPTN is administered by the United Network for Organ Sharing (UNOS) under contract with HRSA. Medicare also requires that hospitals

**Table 40-1.** US Organ Transplants, Waiting Lists, and Adult 3-Year Graft and Patient Survival Rates\*

| Organ               | 2006 Transplants | 2007 Waiting List | Graft Survival Rate (%)  | Patient Survival Rate (%) |
|---------------------|------------------|-------------------|--------------------------|---------------------------|
| Kidney              | 17,090           | 73,233            |                          |                           |
| DD                  | 10,659           |                   | 79                       | 88                        |
| LD                  | 6,431            |                   | 88                       | 95                        |
| Kidney and pancreas | 924              | 2,321             | 85 kidney<br>79 pancreas | 91                        |
| Liver               | 6,650            | 16,714            | 74                       | 79                        |
| Heart               | 2,192            | 2,627             | 79                       | 80                        |
| Lung                | 1,405            | 2,335             | 64                       | 66                        |
| Heart-lung          | 31               | 115               | 53                       | 54                        |
| Pancreas            | 463              | 1,644             | 63                       | 91                        |
| Intestine           | 175              | 215               | 51                       | 62                        |

\*Waiting lists are as of September 2007. Survival rates are for transplantation procedures performed in 2001 with 3 years of follow-up evaluation.<sup>3</sup> Pancreas rates are averages of pancreas alone and pancreas after kidney.

DD = deceased donor; LD = living donor.

report deaths to the 58 organ procurement organizations for evaluation of organ donation eligibility.

As in transfusion medicine, blood group compatibility and disease transmission are of high concern for organ transplants. To reduce the risk of error, OPTN/UNOS policies specify that organ donors and transplant candidates each have two separate ABO typings performed, and the entry of these blood types into the national matching registry must be verified by a second person (OPTN Policies 3.1 and 3.2.4).<sup>6</sup> Medicare regulations also require these measures for organ donors (42 CFR 486.344). ABO errors in labeling or distribution are included in the OPTN/UNOS evaluation plan for assessing compliance with requirements.<sup>7,8</sup>

Organ transplants have transmitted not only blood-borne, transfusion-risk agents, including West Nile virus and *Trypanosoma cruzi* (Chagas' disease), but also other donor infections such as tuberculosis, methicillin-resistant *Staphylococcus*

*aureus*, rabies, lymphocytic choriomeningitis virus, and toxoplasmosis.<sup>9-11</sup> Donor-transmitted cancers occurred in 1 in 12,000 deceased-donor organ transplants in the United States. Although a history of cancer in an organ donor is not necessarily disqualifying, certain tumor types prone to occult metastasis are of extra concern, such as melanoma, lymphoma, and carcinoma of the breast, lung, colon, and kidney.<sup>12,13</sup>

OPTN/UNOS is authorized by federal regulation to establish policies for organ donor screening and testing. Their policies provide a list of infections and medical conditions that, if present, must be communicated to the transplant center. Donors must be tested for hepatitis B surface antigen (HBsAg); antibodies to human immunodeficiency virus (anti-HIV-1/2), hepatitis B core antigen (anti-HBc), hepatitis C virus (anti-HCV), human T-cell lymphotropic virus (anti-HTLV-I/II), cytomegalovirus (anti-CMV), and Epstein-Barr virus (anti-EBV); and a serologic test for syphilis (VDRL or RPR) (OPTN Policies 2.2 and 4.1).<sup>6</sup> The Food and Drug Administration (FDA) maintains a Web page with current information on infectious-disease tests cleared for organ and cadaver (non-heart-beating) donors.<sup>14</sup> These include several nucleic acid tests, although viral nucleic acid testing is not required by OPTN/UNOS. West Nile virus testing is not required, although HRSA issued a notice in 2004 that encouraged screening of living donors near the time of donation.<sup>15</sup>

Cadaver donations providing tissues are also subject to separate FDA tissue transplant safety regulations that focus on reducing the risk of relevant communicable diseases (21 CFR 1271). FDA-cleared cadaveric tests must be used for tissue donors when appropriate and available. Donors are ineligible to contribute organs or tissues if their infectious-disease tests are invalidated by excess hemodilution from recent transfusions or infusions, as defined in organ donor risk factors from the US Centers for Disease Control and Prevention (CDC) (OPTN Policy 4.1.1) and by tissue regulation (21 CFR 1271).<sup>5,6</sup>

OPTN/UNOS policies require prompt investigation of possible organ-borne infections and medical conditions, including look-back to other recipients from suspect donors (OPTN Policy 4.7).<sup>6</sup> A nationwide transplant safety sentinel network with a standardized donor identification system is under development in the United States.

The circulation of a brain-dead donor is maintained until the organs are harvested by transplantation surgeons. Sometimes blood component transfusions are needed for oxygenation and hemostasis. After cadaveric organs are removed, hearts and lungs must be used within 4 to 6 hours, livers within 12 hours, pancreata within 18 hours, and kidneys within 18 to 30 hours.<sup>1</sup> Preservative solutions are used during cold ischemia until transplantation.

## Organ Transplants

The systems for ranking the priorities of patients are specific for each organ and incorporate severity of illness. For each organ

within a region, the patient waiting lists are compiled according to ABO blood group. For each transplant procedure, two to four persons are on the waiting lists for the most commonly transplanted organs (Table 40-1). Organs and organ donors are still scarce. The need for organs has stimulated efforts to expand the supply by using organs from living donors and by splitting livers. Over one-third of US kidney transplants are from living donors.<sup>16</sup> The graft and patient survival rates are better than for renal transplantation from cadavers. A small number of liver transplants (4%) are from living donors, from whom the right lobe is removed. Both the graft split liver and the living donor's liver regenerate to normal size in a few weeks. Cadaveric livers sometimes are split (4%). The right lobe is given to one patient and the rest of the liver to another patient.

## Immunologic Barriers

The fundamental test for organ donation and transplantation is the ABO group, because solid organs almost always must be ABO compatible. Anti-A and anti-B bind to endothelial cells, setting off a cycle of complement fixation, vascular damage, and thrombosis that leads to ischemia and rejection. ABO-incompatible liver transplants are less susceptible to hyperacute rejection than are other organs but the risk of eventual rejection is still high. As group O RBCs are sometimes in shortest supply in relation to demand, so too are group O organs. Group O patients wait longer on average than other patients for nonemergency cadaveric transplantation.

HLA compatibility is also important for most organs, except the liver (see Chapter 57). Nonhepatic transplant candidates are assessed for HLA antibodies and their history of HLA exposure through pregnancy, transfusion, or prior transplant.<sup>6</sup> Recipient HLA antibodies against nonhepatic grafts lead to a much higher risk of acute and chronic rejection. After ABO compatibility, deceased-donor kidneys are first prioritized to recipients matched for all six HLA-A, -B, and -DR antigens, because these matches have slightly better graft survival. Kidneys can also be stored longer than other organs, permitting transport to a matched recipient. Kidney and pancreas transplants, together or separate, generally must be HLA Class I (T lymphocyte) cross-match-compatible before transplantation. OPTN/UNOS policy requires that heart and lung grafts also be prospectively HLA-compatible if the recipient has HLA antibodies or has been exposed. Livers do not require HLA matching or crossmatching. Leukocyte-reduced blood components are discussed below.

## Immunohematology

### Patient Alloantibodies

In the 1980s, 6% to 8% of adult recipients of kidney or liver transplants had clinically significant red cell alloantibodies. Another 8% of liver recipients developed antibodies after the

large number of transfusions given during surgery.<sup>17,18</sup> Both of these patient groups now receive fewer transfusions before and during surgery than in the past. Most patients with renal failure receive erythropoietin. Patients with cirrhosis often are treated to reduce variceal bleeding from portal hypertension with medications to reduce venous pressure ( $\beta$ -blockers, octreotide), sclerotherapy or band ligation of varices, and if necessary, transjugular intrahepatic portosystemic shunting. Consequently, pretransplantation red cell antibody problems are less frequent.

Advance screening for the presence of antibodies to non-ABO antigens is still advisable for candidates for liver transplantation. Some liver transplantation patients have multiple alloantibodies, or alloantibodies to high-frequency antigens; hence, the supply of antigen-negative units is limited. The following strategy has been successful in some programs: 1) give at least the first blood volume of RBCs as compatible, antigen-negative units; 2) after this degree of hemodilution of the antibodies, give unscreened or antigen-positive units; and 3) reserve additional antigen-negative units for after surgery, when transfusions return to small volumes and in anticipation of a possible anamnestic response after immune stimulation.<sup>19</sup>

When Rh-negative patients need large amounts of RBCs during liver or other organ transplantation, it may be necessary to give Rh-positive RBCs. Follow-up tests show that these patients usually do not make anti-D.<sup>20,21</sup> Transplantation immunosuppression greatly reduces the expected high rate of Rh sensitization among patients with normal immune function.

HLA alloantibodies against the donor can cause prompt rejection of renal and cardiac grafts. Candidates for these transplants undergo periodic HLA antibody tests, and then HLA cross-matching against donor lymphocytes to confirm compatibility.

Antibodies from Passenger Lymphocytes

As with hematopoietic stem cell transplants, organ transplants contain lymphocytes that sometimes can make antibodies against antigens in the recipient.<sup>22,23</sup> This is most common when group O organs are placed into non-group-O patients. OPTN/UNOS policies restrict the use of group O deceased-donor organs for non-group-O patients (OPTN Policy 3).<sup>6</sup> In the United States in 2005 there were about 500 excess group O deceased liver donors over the number of group O deceased-donor recipients, indicating that about 9% of deceased-donor liver transplants were from group O donors to non-group-O recipients.<sup>16</sup> In living-donor liver transplants, comparable statistics showed that about 20% of them (60 cases) were group O to non-group-O recipients.

In approximately 40% of liver transplant recipients and 10% of kidney recipients receiving an ABO-unmatched organ, the patient develops IgG antibodies from graft lymphocytes that react against his or her own A or B antigens. A few cases have been caused by group A or B organs in group AB patients. The patient's direct antiglobulin test becomes reactive 1 to 2 weeks after transplantation, and some of these antibodies produce sudden marked hemolysis. The antibodies typically persist for a few weeks. Immunoglobulin allotyping has proved that these

antibodies are of donor origin and are not patient autoantibodies. This phenomenon emerged after azathioprine for immunosuppression was replaced by cyclosporine, which is more permissive for secondary antibody responses, and it has occurred with tacrolimus as well.

Non-ABO red cell antibodies can develop in a similar manner if the organ donor has been alloimmunized against an antigen in the recipient. Rh and other antibodies have been reported, and some have caused temporary hemolysis.<sup>22,23</sup> Women donating kidneys to their children or their partner sometimes transmit pregnancy-induced antibodies. When an organ donor is found to have a clinically significant red cell alloantibody it is prudent to notify the organ procurement agency or in the case of a living donor, the recipient's physician. Then patients may receive RBC transfusions that are negative for the target antigen regardless of whether the patient is antigen positive or antigen negative.

Platelet antibodies have been transferred from organs of a donor who died of cerebral hemorrhage caused by immune thrombocytopenic purpura.<sup>24</sup> Perhaps any antibody-producing cells in the organ donor can be transferred temporarily to the recipient.

Blood Transfusion Needs

Table 40-2 shows recent data for mean blood component usage in nonhepatic organ transplantation. Primary cardiac transplantation without previous cardiac surgery is similar to routine cardiac surgery, but the use of components increases in operations for patients who have undergone previous sternotomy. The use

Table 40-2. Mean Blood Component Usage in Nonhepatic Organ Transplantation

| Organ                                 | Red Blood Cells | Plasma | Platelets | Reference                                         |
|---------------------------------------|-----------------|--------|-----------|---------------------------------------------------|
| Cardiac                               |                 |        |           |                                                   |
| Primary                               | 1               | 1-3    | 1-4       | Prendergast <sup>25</sup><br>Wegner <sup>26</sup> |
| Repeated sternotomy                   | 1-2             | 1-6    | 1-8       | Prendergast <sup>25</sup><br>Wegner <sup>26</sup> |
| After support device (with aprotinin) | 8               | 13     | 12        | Wegner <sup>26</sup>                              |
| Lung                                  |                 |        |           |                                                   |
| Single                                | 2               | 1      | 2         | Triulzi <sup>27</sup>                             |
| Double                                | 6               | 4      | 6         | Triulzi <sup>27</sup>                             |
| Pancreas                              | 2               | 0      | 0         | Nyman <sup>28</sup>                               |
| Kidney                                | 1               | 0      | 0         | Danielson <sup>29</sup>                           |

Units are rounded to the nearest integer. Platelets are expressed as platelet concentrates, with apheresis units counted as 6 units. The cardiac transplantation range is from two centers. In one center, aprotinin reduced overall component transfusions in repeated sternotomy cases in a randomized study.<sup>25</sup>

of aprotinin in the care of patients undergoing second operations reduced overall blood component needs in a randomized study.<sup>25</sup> Patients undergoing transplantation after use of a cardiac support device need large amounts of blood, even when treated with aprotinin, in part because of anticoagulation before transplantation.<sup>26</sup> Double-lung transplantation uses more blood than does single-lung transplantation. Uncomplicated renal transplantation usually is a type-and-screen procedure, as is the living-donor nephrectomy.

## Liver Transplantation

The liver transplantation operation is simple in concept, but serious complications can develop at any time.<sup>30</sup> In the pre-anhepatic first stage, the vascular and biliary connections of the liver are dissected and exposed—portal vein, bile duct, hepatic artery, and inferior vena cava. The liver is separated from the diaphragm and the retroperitoneum, often exposing a raw, bare surface prone to bleeding. In the typical procedure, the major vessels are clamped, the vascular and bile duct connections are transected, and the liver is removed. The first stage usually takes 4 to 8 hours. In the anhepatic second stage, the graft and recipient suprahepatic and infrahepatic vena cava and portal vein are anastomosed end to end. The liver is flushed to remove most of its preservative solution. This second stage usually takes 1 to 1.5 hours. At the beginning of the third stage, the post-anhepatic stage, the large vessels are unclamped to restore portal inflow, and the hepatic artery and bile duct are connected. If all goes well, the third stage is completed in 2 to 4 hours.

Liver transplantation presents a unique set of challenges for transfusion and hemostasis needs. Patients with acute liver failure have low levels of clotting factors. Patients with cirrhosis also usually have thrombocytopenia caused by splenomegaly and low levels of thrombopoietin, much of which is made by the liver. Platelet function may be reduced by liver failure, uremia in hepatorenal syndrome, or anemia. Conversely, some patients have a hypercoagulability syndrome because of high levels of Factor VIII and decreased levels of fibrinolytic proteins.

During the operation, certain problems can arise in each stage. During dissection, portal hypertension can contribute to venous bleeding from the areas around the liver. Before and during the anhepatic stage, fibrinolysis sometimes develops from imbalance of its inhibitors and promoters. The graft is flushed, but when the new liver is unclamped, the patient often is partially anticoagulated by heparin present from the donor's anticoagulation and by heparin-like substances from injured endothelium released into the circulation.

To counteract all the possible problems, the liver transplantation, anesthesia, and laboratory team uses an array of tactics to meet the transfusion and hemostasis needs of the patient. Surgeons use an argon-beam laser to coagulate bleeding surfaces. Two surgical techniques are used in the care of selected patients to improve hemodynamics. If the anatomic features of the graft

are favorable, the liver can be sewn on to the inferior vena cava in a so-called “piggyback” manner to maintain partial blood flow through the vena cava during the operation. Some surgeons use venovenous bypass during the anhepatic stage, either routinely or if interruption of vena caval flow causes too much hypotension. This perfusion technique shunts venous return from the inferior vena cava and the portal vein up to the subclavian vein and improves cardiac output if needed.

To monitor coagulation status and to anticipate transfusion needs, hemoglobin, platelet count, prothrombin time, activated partial thromboplastin time, and fibrinogen are measured regularly. Many programs use thromboelastography (TEG) to supplement routine clotting tests.<sup>31</sup> The TEG instrument measures the initiation, rate, and strength of whole-blood clotting, and displays the results in the operating room as they develop over 20 to 60 minutes. TEG also has the following three functions not available in routine coagulation testing: 1) fibrinolysis can be readily detected; 2) the patient's blood can be tested *in vitro* with protamine or  $\epsilon$ -aminocaproic acid to assess whether these medications would help correct heparinization or fibrinolysis; and 3) hypercoagulability is also observed in some TEG results, but is not well correlated with clinical evidence of thrombosis. TEG testing can be employed in an algorithm for transfusion decision-making.<sup>32</sup>

To meet the patient's transfusion needs, intraoperative blood recovery and reinfusion is helpful.<sup>33</sup> One-third or more of the patient's total RBC transfusions can be provided by recovering shed blood during the operation. The anesthesiologist can give large volumes of warmed blood and fluids through a rapid-infusion pump that delivers more than 1 L per minute if necessary.

Plasma usage usually closely parallels RBC use in operations with large transfusion needs, in effect reconstituting whole blood for the transfusions. If available, whole blood is suitable for the first blood volume or so, because these patients usually have normal levels of Factor VIII. Solvent/detergent-treated (SD) plasma has been used successfully, although it may be deficient in some antifibrinolytic proteins.<sup>34</sup> The SD plasma product in the United States, which is no longer available, was associated with thrombotic or hemorrhagic problems during liver transplant. However, a European product has been used successfully in this setting.

Several programs have reported a range of factors correlating with perioperative transfusion needs. The laboratory variables that appear most often in their conclusions are preoperative anemia, platelet count, prothrombin time (or international normalized ratio), and creatinine.<sup>35</sup> The latter two values, along with bilirubin, are the elements for calculating the model of end-stage liver disease (MELD) score for predicting mortality risk and prioritizing adult transplant candidates in the United States. Higher MELD scores have also been associated with more blood use.<sup>32</sup>

Published blood component usage varies greatly among programs, with recent examples of mean RBC usage ranging from 3 units to 11 to 16 units (Table 40-3). Although there may be some publication bias toward favorable results, patient populations and intraoperative transfusion and surgical practices

**Table 40-3.** Blood Component Usage in Liver Transplants

| Location             | Value  | RBC | FFP | Platelets<br>(doses) | Comments                        | Ref.                     |
|----------------------|--------|-----|-----|----------------------|---------------------------------|--------------------------|
| <b>North America</b> |        |     |     |                      |                                 |                          |
| Los Angeles          | Mean   | 16  | 22  | 1                    | MELD >30                        | Xia <sup>36</sup>        |
|                      | Mean   | 11  | 15  | 1                    | MELD ≤30                        | Xia <sup>36</sup>        |
| Toronto              | Median | 5   | 6   | 0.3                  |                                 | McCluskey <sup>35</sup>  |
| Scottsdale           | Mean   | 3   | 3   | 1                    | TEG-guided, aprotinin           | Frasco <sup>32</sup>     |
| Montreal             | Mean   | 3   | 4   | 0.4                  | Aprotinin                       | Massicotte <sup>37</sup> |
|                      | Mean   | 0.4 | 0   | 0                    | Hypovolemia, aprotinin          | Massicotte <sup>38</sup> |
| <b>Europe</b>        |        |     |     |                      |                                 |                          |
| Budapest             | Median | 12  | 14  | 1                    | Aprotinin, selected cases       | Nemes <sup>39</sup>      |
| Groningen            | Median | 5   | 6   | 0.3                  | Aprotinin                       | Nemes <sup>39</sup>      |
| France               | Median | 5   | 6   | 1                    | Eight centers                   | Ozier <sup>40</sup>      |
| Barcelona            | Median | 2   | NA  | NA                   | Temporary portacaval shunt, 52% | Ramos <sup>41</sup>      |

Median shown when available. Units rounded to nearest integer, except platelets.

RBCs = red blood cells; FFP = fresh frozen plasma; MELD = model of end-stage liver disease score; TEG = thromboelastography; NA = not available.

account for some of the reported variance. In one study, the anesthesiologist was a major determinant. In France the main variable in a national comparison was the institution. Two European centers compared their practices to determine why one had higher blood usage and lower graft and patient survival. They found, as did previous workers, that more bleeding and higher RBC usage correlated with higher mortality. The center with less blood use took more time during surgery for hemostasis, and was more likely to do “piggyback” transplants or venovenous bypass, both of which preserve circulatory hemodynamics when needed. Jehovah’s Witnesses have undergone liver transplantation without blood component transfusions, with careful patient selection and the use of multiple blood conservation strategies.<sup>42</sup>

Liver transplants still require massive transfusion in some cases. Although some of these patients have preoperative coagulopathy, this usually can be corrected with appropriate transfusions in the first stage of the transplantation procedure. The underlying factors in these large hemorrhages usually are intraoperative problems such as unusually severe portal hypertension, cardiopulmonary instability, and anatomic and technical complications causing surgical blood loss. These problems often are not predictable from the patient’s transplantation evaluation, and significant blood use frequently is unexpected.

The role of antifibrinolytic agents in liver transplants has been mixed. When fibrinolysis develops and causes bleeding, tranexamic acid or ε-aminocaproic acid is often utilized. However, opinions vary on whether these drugs or aprotinin should be used as regular prophylaxis, in view of their potential to cause unintended clotting complications. Aprotinin has been used widely in Europe. A meta-analysis of controlled trials concluded that tranexamic acid and aprotinin reduced blood loss without increasing adverse thrombotic events.<sup>43</sup> However, other

reviewers found that aprotinin’s benefits were mainly in patient groups with higher blood loss, and did not recommend routine use.<sup>44,45</sup> Concern has emerged recently about aprotinin and excess mortality after cardiac surgery, and the drug has been suspended by the manufacturer.

Recombinant Factor VIIa was tested in two double-blind randomized trials in liver transplants. In a single-bolus trial, patients received one 20, 40, or 80 µg/kg bolus just before surgery.<sup>46</sup> In the second trial, doses of 60 or 120 µg/kg were repeated every 2 hours.<sup>47</sup> The median transfusion needs were not different from placebo in either study. The role of the drug in selected patients at high risk of bleeding or with refractory coagulopathy is under study.

Even without medications, thrombosis (either systemic or localized to the hepatic artery) is an occasional complication during liver transplant operations. In some patients, this complication is associated with hypercoagulability found during TEG, and moderate heparinization may be advisable. Aggressive correction of coagulopathy should be avoided when clotting would present special difficulties, as during venovenous bypass, or when the hepatic artery is small or not flowing well.

## Special Needs

### Prevention of Cytomegalovirus Infection

Cytomegalovirus infection is a major infectious complication of organ transplantation because of the cellular immunosuppression needed. If a candidate for solid-organ transplantation is anti-CMV-negative, CMV-reduced-risk cellular blood components should be provided (ie, obtained from anti-CMV-negative donors or leukocyte reduced to remove the bulk of CMV viral load).<sup>48</sup> When an anti-CMV-negative transplant recipient

receives an anti-CMV-negative organ, CMV-reduced-risk transfusions are recommended when possible to prevent third-party CMV infection from the blood donor. The growing availability of leukocyte-reduced components has increased the supply of CMV-reduced-risk components for liver transplantation.

For patients who are already anti-CMV-positive or who receive an anti-CMV-positive graft, CMV-reduced-risk transfusions generally are not necessary. The risk of CMV infection comes from the new organ or from reactivation of the original infection. CMV-reduced-risk components do not provide appreciable clinical benefit in these patients (see Chapter 47).

### Leukocyte Reduction

The leukocyte reduction of blood components for organ transplantation presents a paradox. This is one clinical setting in which transfused white cells were historically beneficial.<sup>49</sup> In the 1970s and early 1980s, transfusions were commonly given to candidates for renal transplantation to achieve better graft survival. RBC transfusions leukocyte reduced by means of freezing and thawing did not confer the same advantage. The effect was attributed to immunosuppression by transfused white cells. When immunosuppression improved, the transfusion advantage decreased. However, even with cyclosporine, donor-specific transfusions can impart some benefit for engraftment and survival.<sup>50</sup>

The other side of the paradox is that if the transplant recipient has previous HLA antibodies against the prospective graft kidney, transplantation is contraindicated because rejection of these organs may ensue. Donor-specific transfusions pose a risk of immunization against the donor, preventing the planned transplant. From this perspective, leukocyte reduction of pre-transplant transfusions would be desirable. HLA alloimmunization also adversely affects survival of heart and lung grafts.

The balance of current opinion probably falls on the side of leukocyte reduction to avoid development of HLA antibodies in candidates for nonhepatic organ transplantation. Research continues on transfusion immunomodulation in transplantation (see Chapter 45).

### Blood Irradiation

Graft-vs-host disease (GVHD) has occurred from passenger leukocytes in transplanted organs. However, GVHD from transfusions in these patients is rare.<sup>51</sup> Solid-organ transplantation usually is not included in recommended indications for blood irradiation (see Chapter 54).

### Breaching Immunologic Barriers

#### ABO

Because there are several times more transplant candidates than available annual organ donations, transplant centers have

employed several methods to evade the ABO barrier for selected patients: 1) exchanges of directed organ donors, 2) neonatal tolerance, 3) A<sub>2</sub> organs, and 4) humoral immunosuppression to permit accommodation of fully ABO-incompatible organs.<sup>52,53</sup> Blood bank support is vital to these efforts (Table 40-4).

The majority of kidney transplants are from living donors, but some patients may not have an ABO-compatible donor. In order to obtain ABO-compatible organs, some transplant centers arrange for willing donors to exchange with another patient.<sup>53</sup> Extended chains of such exchanges have been performed. Another strategy is for a donor to contribute a nondirected kidney to the general organ supply, and then the donor's patient of concern receives priority for an ABO-compatible deceased-donor organ.

Infants do not make their own circulating antibodies for several months after birth. This immunologic "loophole" has been successfully exploited for the transplantation of ABO-incompatible hearts in young children.<sup>54</sup> Graft survival is similar to ABO-compatible transplants, and fewer infants die waiting for a heart when ABO is not a limitation. This has been adopted in OPTN/UNOS organ allocation policy, including upper limits on antibody titers in infants 1 to 2 years old (OPTN Policy 3.7.8).<sup>6</sup> The presence of the incompatible ABO antigen at a very young age leads to long-term suppression of antibody production, with absence of donor-specific anti-A or anti-B peripheral B lymphocytes, and low or no anti-A or anti-B against the heart's antigens.

The tissues of persons with the A<sub>2</sub> blood group have weaker group A expression than in persons with the A<sub>1</sub> blood group, about 20% of the normal quantity. Kidneys from group A<sub>2</sub> donors have been successfully transplanted to group O and group B patients, as have group A<sub>2</sub>B kidneys to group B patients.<sup>55,56</sup> In some centers, candidates' anti-A titers are prescreened, and those with high titers are excluded from receiving a group A<sub>2</sub> or A<sub>2</sub>B kidney. However, transplants to eligible low-titer patients have been performed with regular immunosuppression, and results are similar to ABO-compatible grafts.

Fully ABO-incompatible kidney transplants have been performed using protocols with extra immunosuppression, removal of preexisting ABO antibodies by plasmapheresis, blockage of antibody production, and frequent monitoring of ABO antibody levels. These programs are most often established in living-donor transplants, when the date of transplant can be scheduled in advance, in contrast to unscheduled deceased-donor organ

**Table 40-4.** Roles for the Blood Bank in ABO-Incompatible Organ Transplant Protocols

- Seek A<sub>2</sub> organ donors with A<sub>1</sub> lectin or *ABH* gene typing.
- Select or process blood components to avoid giving passive graft ABO antibodies to recipients before and after receipt of transplant.
- Carefully titrate IgM and IgG ABO antibodies.
- Perform plasmapheresis to remove and control ABO antibody levels in pre- and perioperative periods.



transplants, which may not permit extended pretransplant preparations. Japan, which has very few deceased or group A<sub>2</sub> organ donors, has gleaned the most experience. A national registry of 564 ABO-incompatible transplants in 1989-2003 comprised 14% of all living-donor kidneys.<sup>57</sup> With the use of apheresis and splenectomy, the overall 5-year graft survival was 74%, compared to 81% in ABO-compatible controls. Outcomes improved over time, and the most recent 2-year graft survival was 94% for ABO-incompatible transplants.

Recent studies in Japan and other developed countries have sought to replace splenectomy with other means to suppress antibody production, such as rituximab (anti-CD20) and intravenous immune globulin (IVIG) or cytomegalovirus immune globulin (CMVIG) for immunomodulation, and the administration of mycophenolate mofetil to suppress both B and T lymphocytes.<sup>58-60</sup>

In all protocols, plasmapheresis to reduce and control anti-A or -B titers is a central feature. In the current guidelines for therapeutic apheresis from the American Society for Apheresis (ASFA), ABO-incompatible kidney and neonatal heart transplants are Category II indications, generally accepted adjuncts to other treatments, and incompatible liver transplants are in Category III, with suggestion of benefit, but efficacy not established.<sup>61</sup> Several European renal transplant centers have performed plasmapheresis with an in-line column containing A or B antigen to remove ABO antibody by immunoadsorption.<sup>62</sup>

As a corollary to these efforts, recipients or potential recipients of ABO-incompatible organs should not receive transfusions of large quantities of recipient-type plasma containing graft ABO antibody. The transfusion service selects components of appropriate ABO type, or processes them to remove graft antibody before transfusion.

ABO antibody titration is a critical laboratory test for physicians and their patients in these protocols. IgM antibody mediates complement fixation and endothelial damage in acute transplant rejection of ABO-incompatible organs, and IgM is more readily removed by plasmapheresis than IgG. However, many transplant series emphasize IgG titers for patient eligibility, rejection risk, and plasmapheresis guidance. A working group from several US centers recommended reporting both IgM and IgG titers.<sup>63</sup> Antibody titers routinely performed in the blood bank are subject to some degree of imprecision, with a range of  $\pm 1$  dilution (a fourfold difference). However, many transplant protocols set more precise titer targets for eligibility or antibody removal. In Japan, broad inter-institutional variation in titer results on survey specimens was improved with a more standardized method.<sup>64</sup> Future studies may illuminate which titration methods are most clinically useful and reproducible, and determine the role of more quantitative methods for antibody levels.

In the United States, the UNOS registry was examined for the outcomes of ABO-incompatible kidney transplants between 1995 and 2003.<sup>65</sup> With deceased-donor transplants ( $n = 201$ ), the 5-year graft survivals for fully incompatible transplants were the same as ABO-compatible cases (66-67%). For living-donor

kidneys ( $n = 191$ ), the ABO-incompatible grafts had lower overall long-term survival (66% vs 80% at 5 years), although the difference was mainly in the first year.

ABO-incompatible transplants are most vulnerable to rejection in the first month after surgery, and antibody reduction measures focus on this period. However, after the initial risk phase, most grafts survive despite the continuing presence of graft ABO antibody in the plasma and donor-type ABO antigens in the graft endothelium. This survival in the face of humoral immunity has been termed accommodation. Several mediators of rejection might be involved in this phenomenon.<sup>66</sup> Graft ABO antibodies sometimes remain at lower levels than before the transplant procedure, and may change in specificity. In ABO-incompatible kidneys after 3 to 12 months of graft survival, microarray gene expressions of several regulators of inflammation, apoptosis, signal transduction, and cell interaction were significantly altered in ways that suggested injury protection, when compared to ABO-compatible control grafts.<sup>67</sup> Other factors for further investigation include: 1) the effects of graft-derived A and B antigens in plasma, such as those on endothelial-derived von Willebrand factor; 2) complement pathway components and their immunomodulation of the local immune response; and 3) nitric oxide release from ABO-incompatible endothelial cells, which could dampen apoptosis and platelet adhesion.<sup>68</sup>

## HLA

Similar to the ABO blood group, the HLA system poses a significant barrier to transplantation in patients with preexisting antibodies, but immunologic manipulations are making some headway for these patients. Broadly alloimmunized patients are identified by a high percentage of positive lymphocytotoxic crossmatches to Class I antigens on donor or panel T cells. In the United States, 14% of renal transplant candidates have antibodies against  $\geq 80\%$  of potential donors (panel-reactive antibody, PRA).<sup>16</sup> These patients have great difficulty receiving a compatible living or deceased-donor kidney.

Protocols to suppress HLA antibodies have been termed desensitization. The two main alternate strategies currently used are 1) high-dose IVIG, and 2) plasmapheresis and low-dose IVIG or CMVIG.<sup>68</sup> How IVIG works in these protocols is uncertain, but possibilities include receptor blockade, anti-idiotypic antibody content, and inhibition of the activation of lymphocytes and complement.<sup>69</sup>

High-dose IVIG is given at up to four monthly intervals before receipt of living-donor or deceased-donor transplants, until crossmatches are negative, and then once, 1 month after surgery. In-vitro testing to identify candidates in advance has been performed by assessing whether the presence of IVIG inhibits a patient's antibody in lymphocytotoxicity testing. Of 77 sensitized patients passing this prescreening, 87% received living or deceased-donor organs.<sup>69</sup> Rejection episodes occurred in 28%, but the 3-year graft survival was 87%.

Plasmapheresis and low-dose IVIG have been used mainly before living-donor kidney transplants. Multiple plasmapheresis

procedures are performed every other day before and after the transplant procedure (the number of procedures in proportion to the preexisting antibody titer) and IVIG is given after each procedure. The center with the most experience used CMVIG, converted 63% of patients' crossmatches to negative before transplantation, and had approximately 85% 3-year graft survival.<sup>70,71</sup>

A randomized double-blind clinical trial of desensitization compared high-dose IVIG to placebo in 101 renal transplant candidates with PRA  $\geq 50\%$ .<sup>72</sup> The mean PRA was reduced from about 75% to 60% in the treatment group during four monthly infusions, and 35% of treatment patients received a kidney, compared to 17% of controls. For the transplants, the 2-year graft survival rates of 75% to 80% were similar in the two groups.

One center reported comparative data from periods when either high-dose IVIG or plasmapheresis/IVIG plus rituximab were used. The plasmapheresis/IVIG patients were desensitized in 85% of 48 cases, compared to 38% of 13 high-dose IVIG cases.<sup>73</sup>

Antibody-mediated rejection has also been treated with either of these two approaches, as well as rituximab alone.<sup>74</sup> In the ASFA guidelines, plasmapheresis for HLA desensitization and antibody-mediated rejection are both Category II indications, generally accepted adjunctive therapy.<sup>61</sup>

### Other Applications of Therapeutic Apheresis

Photopheresis is under study for the prevention and treatment of heart and lung transplant rejection.<sup>75</sup> Leukocytes are collected, exposed to psoralen and ultraviolet A light *ex vivo*, and then reinfused. This is thought to induce immunomodulation of T cells regulating or mediating organ rejection. ASFA classifies this therapy in Category III (suggestion of benefit, insufficient evidence of efficacy) for treating lung rejection manifesting as bronchiolitis obliterans.<sup>61</sup> Also under Category III is plasmapheresis in acute liver failure, as a supportive therapy until hepatocellular recovery or transplantation.<sup>61</sup>

## Artificial Organs

Several types of cardiac assist devices are in use or under development for the care of patients with severe cardiac failure.<sup>76</sup> Some are for temporary use or as a bridge to cardiac transplantation. Others are intended for permanent implantation. While using these devices, patients often develop coagulopathy from platelet dysfunction or anticoagulation. Transfusions during these treatments can cause HLA alloimmunization, and leukocyte-reduced components should be used if transplantation is planned.

Hepatic assist devices are perfused with the patient's blood or plasma to remove toxins.<sup>77</sup> These systems are in clinical trials to support patients until their liver recovers, as in poisoning, or until a liver transplant is available. One category of these systems passes the blood through various absorbent materials such as charcoal, resins, or albumin. The other type metabolizes toxins in the blood with immobilized porcine or human hepatocytes in a perfused bioreactor.

## Summary

As organ transplant graft and patient survivals continue to gradually improve, the limited supply of organs has stimulated many creative ways to hurdle traditional immunologic barriers of ABO and HLA tissue incompatibility. The role of transfusion medicine has expanded beyond transfusions to include the laboratory and clinical support of therapies to prevent and reverse graft rejection, making transplants more available and more durable. Progress in this field may lead to new advances in other diseases when immune reactions are foe, not friend.

## Disclaimer

The author has disclosed no conflicts of interest.

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# III    Apheresis

# 41

## Apheresis: Principles and Technology of Hemapheresis

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*Apheresis*, a word of Greek derivation, means “removal” in its broadest sense. In this chapter, it refers to any procedure during which blood is withdrawn from the donor or patient and separated *ex vivo* into some or all of its components. Some of these components are retained for donation or therapeutic purposes. The others are returned, usually “on line,” to the person. The word *pheresis* once was used synonymously with the word *apheresis*; however, *apheresis* is the preferred word. *Hemapheresis* is frequently used synonymously with *apheresis*.

### Historical Background

The first experimental apheresis procedure was performed in 1660 by Richard Lower of Oxford, England, who performed a manual procedure on dogs. Plasmapheresis (removal of plasma with return of red cells) was first performed in France in 1902 and in Russia in 1914.<sup>1</sup> In 1914 at Johns Hopkins University, Roundtree and Turner used plasmapheresis in artificial kidney research.<sup>1</sup>

In 1960, Soloman and Fahey used manual plasmapheresis therapeutically to reduce elevated globulin levels in a patient with a hyperviscosity syndrome, and thus began the era of therapeutic apheresis.<sup>1,2</sup> Apheresis became a practical reality with technologic advances, which included development of plastic bags, integrally connected tubing, and *ex-vivo* centrifugation. Initially, apheresis involved blood separation as an “off-line” disconnected process in laboratories with freestanding component separation centrifuges. Now, apheresis is routinely performed as an “on-line” procedure with fully automated blood cell separators at the donor’s or patient’s bedside.

Manual donor plasmapheresis with paid plasma donors was the major method of collecting Source Plasma for fractionation into plasma derivatives (albumin, immunoglobulins, Factor VIII,

and Factor IX) between 1950 and 1980. Because the process was “off line,” requiring separation of the donor from the unit of whole blood during plasma separation, the possibility of returning the red cells to the wrong donor existed and occasionally did occur. The long time of 1.5 to 2 hours to collect 500 to 700 mL of plasma led to the use of paid donors and ultimately to the contamination of many plasma derivatives with hepatitis B virus (HBV) and hepatitis C virus (HCV), and human immunodeficiency virus (HIV). In the 1980s, automated on-line cell separation devices were developed by Haemonetics (Braintree, MA), Baxter Fenwal (Deerfield, IL), and Organon Teknika (Boxtel, The Netherlands) for collection of Source Plasma in the United States and Europe. By 1985, an estimated 120,000 to 150,000 automated donor plasmapheresis procedures had been performed in the United States; however, 15 years later that number was more than 12 million a year and now is estimated to exceed 17 million donations per year worldwide.<sup>3</sup>

Granulocytes, platelets, and Fresh Frozen Plasma (FFP) were the original components collected by means of apheresis technology. Today, numerous blood components are collected from a single donor by means of automated apheresis technology, including the following:

1. Platelets.
2. Plasma.
3. Red Blood Cells (RBCs).
4. RBCs plus plasma (RBCP).
5. 2-unit RBCs (2RBCs).
6. Platelets and RBCs.
7. Platelets and plasma.
8. Granulocytes stimulated with steroids or colony-stimulating factors.
9. Hematopoietic progenitor cells (HPCs) stimulated with granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF).

In the past few decades, plateletpheresis procedures have increased dramatically—from 171,200 in 1989 to 1,167,000 in 2006.<sup>4</sup> RBC apheresis procedures, especially 2RBC collections, are rapidly increasing. Nationally, the collection of apheresis RBCs increased from

824,000 in 2004 to 1,619,000 in 2006.<sup>4</sup> Of the 149,763 collections of allogeneic RBCs at the Oklahoma Blood Institute (OBI) in 2000, 13,944 (9.3%) resulted from RBC apheresis procedures. In 2006, the apheresis RBCP or 2RBC procedures at OBI produced 24,685 units (13% of total RBC units).

Therapeutic apheresis procedures (therapeutic plasma exchange and therapeutic cytapheeresis) have increased as well—from an estimated 60,000 to 80,000 in 1985 to more than 112,000 in 2006.<sup>4</sup> One reason for the increase is the use of hemapheresis technology to remove red cells provides a new approach for patients with hemochromatosis. Another reason is the expanding role of photopheresis in managing conditions such as graft-vs-host disease (GVHD) and graft rejection.

## Current Technology Overview

Edwin J. Cohn, originator of plasma fractionation processes to produce plasma derivatives such as albumin and  $\gamma$ -globulin, developed a prototype machine for centrifugal separation of the cellular elements of blood from plasma. Allen “Jack” Latham, cofounder of Haemonetics, working with Cohn’s prototype, developed a disposable polycarbonate plastic bowl with a rotary seal, which was first used to collect platelets in 1971. Concurrently, George Judson, an IBM engineer on loan to the National Cancer Institute, developed another machine to facilitate white cell (granulocyte) collection.<sup>1</sup> Plasmapheresis and eventually therapeutic plasma exchange (TPE) and therapeutic

cytapheeresis were natural developments in the use of these early apheresis devices to manage human diseases known or thought to be mediated by plasma or cellular factors.

The apheresis devices currently approved by the US Food and Drug Administration (FDA) for donor and patient (therapeutic) use are outlined in Table 41-1. These devices are either discontinuous flow (Haemonetics) or modified continuous flow [Fenwal (Lake Zurich, IL), Fresenius (Bad Homburg, Germany), CaridianBCT (Lakewood, CO)] systems that can be used for one- or two-arm venous access for apheresis procedures. The spinning membrane technology used in the Autopheresis-C (Fenwal) and the flexible bowl technology of Transfusion Technologies (acquired by Haemonetics in 2000) are unique. The latter device has an expandable membrane over a flat disk. Fluid pressure controls the shape and volume of the collecting apheresis chamber and offers unique options for future blood component collection and separation. This flexible bowl is being used in an intraoperative and postoperative autotransfusion device (Haemonetics OrthoPAT System) and in the Haemonetics Cymbal Automated Blood Collection System currently approved for 2RBC collection.

### Fenwal Technology

Fenwal uses continuous-flow technology and a sealless system in the CS3000 blood cell separator, originally introduced in the early 1980s. The device is fully automated and computer controlled. The CS3000 concentrates platelets during collection into a final volume of approximately 200 mL. An enhancement of the CS3000 with a platelet-collection chamber called the TNX-6

**Table 41-1.** Apheresis Devices Used in the United States for Donor Collection and Patient Therapeutics

| Manufacturer/Device | Type  | Product/Procedure     | Venous Access | Intended Use     |
|---------------------|-------|-----------------------|---------------|------------------|
| <b>Fenwal</b>       |       |                       |               |                  |
| CS-3000             | C/CF  | PLAP, HPC, TPE        | 1 or 2 arms   | Donor or patient |
| Amicus              | C/CF  | PLAP, AAFP            | 1 or 2 arms   | Donor            |
| ALYX                | C/CF  | 2RBC, RBCP            | 1 arm         | Donor            |
| Autopheresis-C      | SM/DF | AAFP, SP              | 1 arm         | Donor            |
| <b>Fresenius</b>    |       |                       |               |                  |
| AS104               | C/CF  | PLAP, HPC, TPE        | 1 or 2 arms   | Donor or patient |
| <b>CaridianBCT</b>  |       |                       |               |                  |
| Spectra             | C/CF  | PLAP, HPC, TPE        | 1 or 2 arms   | Donor or patient |
| Trima/Trima Accel   | C/CF  | PLAP, AAFP, RBC, 2RBC | 1 arm         | Donor            |
| Optia               | C/CF  | TPE, HPC              | 1 or 2 arms   | Patient          |
| <b>Haemonetics</b>  |       |                       |               |                  |
| MCS+(LN9000)        | C/DF  | PLAP, AAFP, TPE       | 1 or 2 arms   | Donor or patient |
| MCS+(LN8150)        | C/DF  | RBCP, 2RBC            | 1 arm         | Donor            |
| Cymbal              | C/DF  | 2RBC                  | 1 arm         | Donor            |
| PCS-2               | C/DF  | AAFP, SP              | 1 arm         | Donor            |
| <b>Therakos</b>     |       |                       |               |                  |
| UVAR-XTS            | C/DF  | PCWBC                 | 1 arm         | Patient          |

C = centrifugal; CF = continuous flow; DF = discontinuous flow; SM = spinning membrane; PLAP = platelet by apheresis; AAFP = apheresis fresh frozen plasma; RBC = red blood cell; 2RBC = 2-unit red blood cells; TPE = therapeutic plasma exchange; SP = source plasma; HPC = hematopoietic progenitor cells; PCWBC = photochemically modified white blood cells.

separation chamber has decreased the white cell content in the platelet product. The CS3000 Plus can be used to collect granulocyte-platelet concentrates and HPCs. These devices are being slowly removed from the market.

Fenwal has developed a newer cell separator called the Amicus, which operates in a manner similar to the CS3000, but has a smaller footprint and operates as a one-arm venous access device for platelet collection. The high extraction coefficient for platelets allows frequent collection of double products, such as two apheresis platelet products each containing at least  $3.0 \times 10^{11}$  platelets and less than  $1.0 \times 10^6$  residual leukocytes without the use of a leukocyte reduction filter. In donors with platelet counts of  $\geq 250,000/\mu\text{L}$  before donation, double plateletpheresis products are obtained more than 75% of the time.

The ALYX is a relatively new and compact mobile automated blood collection system that was initially developed as a 2RBC apheresis collection device. It has been approved for collection of concurrent plasma. Following collection in the rigid, cylindrical centrifuge chamber, red cells are pumped through an on-line leukocyte reduction filter into the final storage bag(s).

Fenwal also uses a device called the Autopheresis-C with a spinning membrane, thus incorporating both membrane and centrifugation technology to collect plasma. In the United States, this device is commonly used to collect Source Plasma as well as apheresis FFP. This is a discontinuous system in which only a single venipuncture is performed. Fenwal attempted to change the porosity of the membrane to collect platelet-rich plasma,<sup>5</sup> but the procedure has been abandoned.

### Fresenius Technology

Fresenius introduced a cell separator in Europe in 1987 primarily for the collection of platelets. The Fresenius AS104 is currently licensed in the United States for the collection of apheresis platelets and is licensed for TPE and collection of HPCs. It is a continuous-flow, double-venipuncture system. Fresenius has introduced an upgraded version called the AS204 in Europe but not in the United States. Further development led to the COM.TEC, the latest commercialized version in Europe and Canada. It is not yet available in the United States.<sup>6</sup>

### CaridianBCT Technology

CaridianBCT (formerly Gambro and COBE) acquired the IBM biomedical services technology (the IBM-2997 cell separator) and then developed the COBE Spectra as a sealless system based on the original IBM-2997 rotating-channel belt. The Spectra is a continuous-flow system in which one- or two-arm venous access is used for platelet collection. The Spectra uses a leukocyte reduction system to collect leukocyte-reduced platelet units. The Spectra device indicates when units contain excessive numbers of leukocytes and thus when counting is needed to document leukocyte reduction status. The Spectra device also is used to collect HPCs and to perform therapeutic apheresis procedures.

The newest CaridianBCT cell separators are the Elutra Cell Separation System (not currently FDA-approved) and the

Spectra Optia Apheresis System (recently FDA-approved). The Elutra Cell Separation System is an “off-line” system that uses counterflow centrifugal elutriation to separate cell populations. The Spectra Optia Apheresis System is based on the Spectra but uses a Trima platform and is aimed at the therapeutic apheresis and cell therapy markets. This instrument will eventually incorporate the multiple programs from the Spectra, but in the more portable size of the Trima platform. At the present time it is approved only for TPE.

The Trima system has evolved into the Trima Accel Collection System and is also a relatively new device. The original Trima device is fully automated; the technology is similar to that of the Spectra device. The Trima is a one-arm venous access device, has no rotating seal, and has a smaller footprint than does the Spectra. The Trima is capable of collecting double units ( $\geq 6.0 \times 10^{11}$  platelets) that are leukocyte reduced to  $1.0 \times 10^6$  or fewer residual white cells. The Trima has been designed and is FDA approved to collect a single or double RBC unit along with the apheresis platelet product. To date it has not been used for therapeutic procedures. Collection of 1 or 2 RBC units with a platelet unit ( $\geq 3.0 \times 10^{11}$  platelets) that is leukocyte reduced is a novel idea and has the advantage of increasing the RBC supply of a blood center. The disadvantage is the possibility of deferring a donor on the next visit if the donor's hematocrit decreases to less than 38%. Because of the low extracorporeal red cell mass in the Trima device, a donor giving red cells and a plateletpheresis product can return within 72 hours to donate another plateletpheresis product with the Trima device—provided the donor's hematocrit is 38% or higher.<sup>7</sup> For donors with a predonation platelet count of  $\geq 250,000/\mu\text{L}$ , double plateletpheresis products can be collected more than 75% to 80% of the time.

The Trima Accel Collection System collects platelets, red cells, plasma, and combinations of these blood components. It is unique in that it uses the single-channel belt, whereas the original Trima used the dual-channel belt for the collection of platelets. By using the single-channel belt coupled to an internal leukocyte reduction system, the Trima Accel has an increased platelet extraction coefficient and still maintains a low residual white cell count of less than  $10^6$  per component. The advantages of the Trima Accel are 1) it can collect the same number of platelets as the “standard” Trima but in a shorter time, 2) it can collect more platelets in the same time as the “standard” Trima, or 3) it can collect the same number of platelets in the same time as the “standard” Trima, but do it by processing less of the donor's blood, which means delivering less citrate to the donor and reducing citrate-induced donor reactions.

Obsolete CaridianBCT technologies include a continuous-flow device with a flat membrane for blood separation and the COBE/IBM 2997.

### Haemonetics Technology

The discontinuous-flow cell separators (MCS+ LN9000; MCS+ LN8150; PCS-2) produced by Haemonetics operate with a fixed centrifuge speed and a variable centrifugal force to separate a



donor's or patient's blood into components. A disposable hard-shelled rotating bowl separates the incoming blood in such a way that red cells move to the periphery and plasma to the inside of the rotating bowl. The buffy coat, which contains the white cells and platelets, forms a layer between the red cells and plasma. Platelets or mononuclear cells are collected with the aid of optical detectors by means of a fluid-surge elutriation process. The remaining red cells and plasma are returned to the donor, and the platelets are retained until the desired yield is obtained with multiple passes or cycles. This discontinuous technology is amenable to a one- or two-arm protocol, although the one-arm venous access technique is used more often because of donor preference for a single needlestick.<sup>8</sup> The Cymbal Automated Blood Collection System uses the Dynamic Disk, which is a flexible expandable variable volume bowl for the collection of 2RBC as currently FDA approved. It is also a discontinuous flow system.

The Haemonetics plasma collection system-2 (PCS-2), which currently does not have a functionally closed rotating seal, has a disposable bowl specifically designed to collect plasma. This has become the most commonly used automated cell separator for collection of Source Plasma in the United States and the world. The PCS-2 also is used to collect transfusable plasma in clinically useful volumes of 500 to 600 mL (apheresis FFP). The MCS+ cell separators are fairly lightweight [60 to 70 lb (27 to 31.5 kg)], extremely portable and mobile, fully automated, and flexible in terms of blood components collected. The MCS+ LN9000 is used strictly to collect platelets with or without concurrent plasma. Unlike the Fenwal Amicus and Caridian Trima or Spectra leukocyte reduction system, the initial platelet product is not leukocyte reduced to an acceptable level ( $\leq 1.0 \times 10^6$  residual white cells) and must undergo leukocyte reduction via filtration, which is now accomplished with an on-line process that produces a final transfusable product that contains  $\leq 1.0 \times 10^6$  residual white cells in  $3.0 \times 10^{11}$  or more platelets.

The MCS+ LN8150 has been developed for collection of either RBCP or 2RBC.<sup>9-11</sup> Each RBC unit contains approximately 180 to 200 mL of absolute red cell mass with the machine preprogrammed for the desired amount of red cell mass. Both MCS+ devices (LN9000 and LN8150) have a functionally closed rotating seal to maximize product shelf life. The MCS+ LN8150 operates with an anticoagulant to anticoagulated whole blood (AC:ACWB) collection ratio of 1:16 with citrate-phosphate-dextrose-dextrose (CP2D), which further reduces the amount of citrate in the RBCP or 2RBC units but markedly reduces the citrate in the plasma collected. The plasma of the RBCP collection contains one-half the citrate of whole-blood-derived (WBD) FFP. The red cells from the 2RBC procedure can be filtered on line using a single filter to yield 2 leukocyte-reduced RBC units. These systems return saline to minimize the chance of donor hypovolemia.

The newest instrument/system available from Haemonetics is the Cymbal Automated Blood Collection System, a small, lightweight, portable, discontinuous flow device intended for the collection of 2 RBC units. This device uses a variable volume

bowl that expands as blood fills it, with separation occurring based on density in the centrifugal field. The system uses CP2D with AS-3 and filters the red cell on line to achieve leukocyte reduction.

### Therakos (Johnson & Johnson) Technology

The newest photopheresis device (UVAR-XTS system) is a discontinuous-flow cell separator that collects buffy coat containing mononuclear cells from patients with a variety of conditions such as cutaneous T-cell lymphoma, GVHD resulting from marrow transplantation, or solid-organ rejection. Methoxsalen (Uvadex) is injected into the buffy coat product, the cells are incubated and irradiated with ultraviolet light, and the component is transfused back to the patient. Amelioration of the pathologic process through immunologic effects on clones of mononuclear cells is the desired outcome. The older Therakos UVAR photopheresis system used oral administration of methoxypsoralen and is no longer supported.

### Other Technology

Other devices, both centrifugal and membrane based, are used outside the United States. Dideco (Mirandola, Italy) makes an automated blood cell separator (Excel) for the collection of apheresis platelets. Organon Teknika developed a hollow-fiber membrane (Plasmapur) plasma collection system, which was previously used in Europe to collect source plasma. Several Japanese and European manufacturers make hollow-fiber separators and filters for donor and therapeutic use. Immunoabsorption and cellular adsorption technologies continue to be tried for removal of pathologic antibodies and to modify cell-mediated immunity respectively, although most have been withdrawn from, or are not readily available in, the US market.<sup>12</sup> Several methods have been developed for removal of lipid fractions from patients with homozygous type II hypercholesterolemia. These technologies (see Chapter 43) are expensive and for the most part rather complicated, but effective in a select group of patients.<sup>13,14</sup>

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## Donor Apheresis

### General Information

The key forces driving transfusion medicine in the early 21st century are donor and recipient safety enhancement, improved product quality, error reduction, blood availability, legal and regulatory issues, and cost. The issues of safety, quality, and error reduction with regard to blood components are enhanced with the use of apheresis-derived products. Metered anticoagulation, automation during collection, reduced variables, labeling techniques, and fewer donor exposures all enhance safety, quality, and error reduction. The issues of blood availability (especially RBC units) and increasing cost (especially for testing for infectious and noninfectious agents and additional processes such as leukocyte reduction) have become critical issues. Apheresis components that have been more expensive to produce in the past are now being produced as

double- and triple-yield products or as multiple types of products (RBCP and RBCs plus platelets). This reduces costs while increasing availability. The advantages of apheresis-derived blood components are listed in Table 41-2. Blood components that can be obtained are presented in Table 41-3.

### Donor Care

Donors who undergo automated apheresis must go through routine screening and testing procedures. At minimum, apheresis

**Table 41-2.** Advantages of Apheresis-Derived Blood Components

- Reduced donor exposure: full transfusion dose
- Frequent repeat donor: “pedigreed” donors
- Higher quality products: more quality control per component collected
- Consistent and standardized products (yields)
- Matching donors to patients
- Reduced donor reactions
- High donor acceptance
- Double yield or multiple full-dose blood component collections
- Safety enhancement: for the patient

**Table 41-3.** Apheresis-Derived Blood Components from a Single Procedure

| Procedure          | Instrument                                                     |
|--------------------|----------------------------------------------------------------|
| <b>Primary</b>     |                                                                |
| PLAP               | MCS+(LN9000), Spectra, Trima/TrimaAccel, CS3000, Amicus, AS104 |
| PLAP + Plasma      | MCS+(LN9000), Spectra, Trima/TrimaAccel, CS3000, Amicus AS104  |
| PLAP + RBC         | Trima/Trima Accel                                              |
| 2RBC               | MCS+(LN8150), Cymbal, ALYX                                     |
| RBCP               | MCS+(LN8150), ALYX                                             |
| Plasma             | PCS-2, Autopheresis-C                                          |
| Granulocytes       | MCS+(LN9000), Spectra, CS3000,                                 |
| HPC                | Spectra, Optia, CS3000, AS104                                  |
| <b>Secondary</b>   |                                                                |
| Cryoprecipitate    | PCS-2, Autopheresis-C                                          |
| Cryoreduced Plasma | PCS-2, Autopheresis-C                                          |

PLAP = plateletpheresis; RBC = red blood cells; 2RBC = 2-unit red blood cells; RBCP = red blood cells plus plasma; HPC = hematopoietic progenitor cells.

component donors must meet all the requirements of whole-blood donors. The main difference is that the “needle in to needle out” time for a 500-mL whole-blood donation is only 8 to 12 minutes, whereas the time for plasmapheresis and red cell apheresis is 35 to 45 minutes, and the time for plateletpheresis is 60 to 120 minutes. The longer total time for apheresis donation requires more precise scheduling and a more dedicated donor. When 2 RBC units are donated, the interval between donations is 112 days, for a maximum of three donations per year. For whole blood donors, the maximum is six donations per year. Plateletpheresis donors can give up to 24 donations per year. Donors of transfusable apheresis plasma can make up to 13 donations per year (every 28 days) if the blood center obtains a variance from the FDA with regard to regulations for Source Plasma donors, who donate more frequently. The blood center plasma donors are known as infrequent donors under this variance.

In the United States, Source Plasma (for fractionation into albumin, intravenous immune globulin, Factor VIII, and Factor IX) is obtained almost exclusively from compensated donors who donate at commercially operated plasma centers. These donors may donate up to 800 mL of absolute plasma per donation as frequently as twice a week, depending on the donor's weight. The donor must be examined at defined intervals by a physician and must have total protein and serum protein electrophoresis determinations made at FDA-required intervals as specified in the *Code of Federal Regulations*.<sup>15</sup>

Apheresis donors tend to have lower reaction rates than do donors of whole blood (Table 41-4).<sup>16</sup> Vasovagal reactions are most common among first-time blood donors; apheresis donors usually are repeat donors.<sup>17</sup> Hypovolemia is less common among apheresis donors than among whole-blood donors for two reasons. First, the total volume removed in apheresis donation procedures is less than that in whole-blood donation because the component has a lower volume, as in plateletpheresis, or volume is returned to the donor as crystalloid solution in the anticoagulant and saline solution. Second, the relatively long time at rest during apheresis donation allows transcapillary refilling of the intravascular compartment from the interstitial space. Allergic reactions to iodine skin preparations—the reaction is to the organic radical attached to the iodine—are very rare. However, a mild citrate

**Table 41-4.** Donor Reactions

| Reaction       | Whole Blood  | Apheresis FFP | Apheresis Platelets | 2RBC, RBCP |
|----------------|--------------|---------------|---------------------|------------|
| Vasovagal      | Occasionally | Rare          | Rare                | Rare       |
| Hypovolemia    | Occasionally | Rare          | Rare                | Rare       |
| Allergic       | Very rare    | Very rare     | Very rare           | Very rare  |
| Citrate effect | None         | Rare          | Frequent            | Rare       |
| toxicity       | None         | Very rare     | Occasional          | Very rare  |

FFP = fresh frozen plasma; 2RBC = 2-unit red blood cells; RBCP = red blood cells plus plasma; Occasional = 0.5% to 2.5%; Rare = <0.5%; Very rare = <0.01%; Frequent = 5% to 20%.

effect (circumoral paresthesia, feelings of vibration or buzzing, tingling, or coldness) is not uncommon for most plateletpheresis donors. More severe citrate toxicity (muscle cramping, total body shivering, nausea, vomiting, and tetany) is uncommon but is potentially serious and necessitates slowing the return rate of citrate-containing plasma.<sup>18,19</sup> The intravenous administration of calcium-containing solutions is generally not recommended, unless the reactions are unusually severe or prolonged.

Hemolysis occurs rarely and when it does, the cause is almost always mechanical, such as occlusion or partial occlusion of red-cell-containing tubing. On rare occasions (1 in 3000 to 5000 donations), hyperlipemic plasma in platelet donors causes elevated and visible free hemoglobin levels in the collected component. The mechanism of this hemolysis remains unclear.

Venous access is an important consideration in donor apheresis procedures because of the need for 1) long “needle in to needle out” time, 2) a prolonged high flow rate, 3) increased frequency of donation, and 4) the occasional need for two venipunctures with continuous-flow equipment. Possible venous access injuries include blood infiltration and bruising and, rarely, venous thrombosis. Cutaneous nerve injuries are rare, and deep nerve injuries (median, ulnar, and radial nerves) are almost nonexistent. The development of smaller-needle technology for venous access will help reduce these problems.

Extracorporeal blood volume (ECBV) in apheresis procedures is greatly reduced with use of newer devices, such as the Amicus and Trima. With the older devices, especially the discontinuous-flow devices, ECBV tends to be greater but generally is not a problem except in therapeutic apheresis involving pediatric patients. The supine position of donors and access to oral fluids during the procedure eliminate most problems. However, when the older, higher ECBV devices are used, malfunction of a machine can lead to loss of ECBV, resulting in a 56-day donor deferral. The low ECBV of the newer devices allows another plateletpheresis procedure within 3 days if necessary. If the ECBV of the apheresis device is 100 mL or less, plateletpheresis may be performed less than 8 weeks after a whole-blood donation.<sup>20(p24)</sup>

In the early years of donor apheresis, lymphocyte depletion with loss of immunologic memory was a concern because of high white cell levels in apheresis components, especially in platelets. This has proved not to be a problem, and with the current focus on leukocyte-reduced apheresis components, it is no longer a concern even for very frequent apheresis donors.<sup>21</sup> Frequent plateletpheresis does not clinically decrease platelet counts in donors.<sup>22</sup>

### Specific Products and Procedures

Plateletpheresis donations are limited to 24 per year by the AABB and the FDA. Plateletpheresis donation can occur every 72 hours but not more than twice a week. If the interval between plateletpheresis donation is 4 weeks or more, a predonation platelet count is not required. If the interval is less than 4 weeks, a predonation platelet count must be obtained and must be  $\geq 150,000/\mu\text{L}$  for the procedure to be performed. If red cell loss during the procedure exceeds 200 mL, the waiting period is 8 weeks before the next

donation. Donors with predonation platelet counts of  $\geq 300,000/\mu\text{L}$  usually can donate a double-dose platelet product ( $\geq 6.0 \times 10^{11}$  platelets). Almost all plateletpheresis products in the United States are produced as leukocyte reduced (meeting AABB standards<sup>20(p37)</sup> for  $5.0 \times 10^6$  or fewer residual leukocytes). However, the European standard is  $1.0 \times 10^6$  or fewer residual leukocytes per product. Prestorage leukocyte reduction can be accomplished “in process” (nonfiltration leukocyte reduction, CaridianBCT and Fenwal) or immediately after (filtration, Haemonetics) for virtually all plateletpheresis procedures. Prestorage leukocyte reduction has clear advantages over poststorage or bedside leukocyte reduction filtration in reducing febrile reactions and other leukocyte-related complications by preventing accumulation of cytokines and leukocyte fragments during storage.<sup>23-25</sup>

The intravascular volume deficit during the procedure should not exceed 10.5 mL/kilogram of the donor’s weight. Plateletpheresis units must contain  $3.0 \times 10^{11}$  or more platelets per unit in at least 75% of the units tested at maximal storage time. The physician in charge of the donor apheresis unit can make a medical decision to accept a donation from someone not eligible at the time if the benefits to the intended recipient outweigh the risks to the donor. This can occur when the donor is an HLA match or is HPA-1a (Pl<sup>A1</sup>) negative and no other donor is available. Use of single venous access and the high platelet extraction coefficients in the newer plateletpheresis devices (Amicus, Trima Accel) allow shorter collection times or higher product yields in normal collection times.

Automated red cell collection with apheresis technology can be performed using the Haemonetics MCS+ LN8150, which can collect 2 units of RBCs (2RBC) or 1 RBC unit and 1 large-volume plasma unit (RBCP), or the Caridian Trima, which can collect 1 plateletpheresis product and 1 or 2 RBC units.<sup>10,11,26</sup> The Fenwal ALYX and Haemonetics Cymbal have also been designed for collection of 2 RBC units. The 2RBC procedure is becoming increasingly popular in the United States to collect group O, Rh-negative RBC units with the intent of increasing RBC availability. The advantages are 1) a standardized red cell mass (180 to 200 mL per unit), 2) metered anticoagulation, which obviates mixing and reduces clot formation, 3) the ability to return fluids to the donor and reduce any risk of hypovolemia, 4) on-line separation of RBCs and plasma as either a 2RBC or RBCP product, eliminating secondary separation procedures, and 5) the use of smaller needles. For the collection of 1 RBC unit along with another component (plasma or platelets) the standard hematocrit criteria apply. However, for 2RBC collection, FDA has imposed specific donor hematocrit, height, and weight requirements.<sup>11</sup> Table 41-5 outlines the current criteria for allogeneic 2RBC collection as defined by the FDA. The use of 2RBC collection from female donors is limited. After 2RBC apheresis donation, donor deferral for any procedure (whole blood or apheresis) is 16 weeks. The RBCP criteria for collection of 1 unit of RBCs and 1 unit of jumbo plasma are outlined in Table 41-6. The anticoagulant used in RBCP and 2RBC procedures with the Haemonetics MCS+ LN8150 is CP2D at an

AC: ACWB ratio of 1:16. This markedly reduces (50%) the citrate returned to the donor during these procedures as well as the citrate in the plasma collected. The absolute volume of plasma collected (about 90%) is higher than that derived from a whole-blood collection (approximately 80% of which is absolute).

Automated collection of plasma can be accomplished with all of the apheresis devices, either as a sole collection product (Fenwal Autopheresis-C and Haemonetics PCS-2) or as a concurrent product with apheresis platelets or as part of an RBC apheresis procedure (RBCP). Table 41-6 shows the volume of plasma that can be

**Table 41-5.** Criteria for Allogeneic Donation of 2-Unit Red Blood Cells by Apheresis

| Donor Weight (lb) | Donor Height (in) | Donor Hematocrit (%) | Maximum Absolute Red Cell Volume (mL) |
|-------------------|-------------------|----------------------|---------------------------------------|
| <b>Men</b>        |                   |                      |                                       |
| 130-149           | ≥61               | ≥40                  | 180 × 2                               |
| 150-174           | ≥61               | ≥40                  | 200 × 2                               |
| ≥175              | ≥61               | ≥40                  | 210 × 2                               |
| <b>Women</b>      |                   |                      |                                       |
| 150-174           | ≥65               | ≥40                  | 180 × 2                               |
| ≥175              | ≥65               | ≥40                  | 200 × 2                               |

**Table 41-6.** Criteria for Allogeneic Donation of Red Blood Cells and Plasma by Apheresis

| Donor Weight (lb) | Donor Hematocrit (%) | Max Absolute Red Cell Volume (mL) | Max Absolute Plasma Volume (mL) |
|-------------------|----------------------|-----------------------------------|---------------------------------|
| <b>Men</b>        |                      |                                   |                                 |
| 110-129           | ≥38                  | 185                               | 450                             |
| 130-149           | ≥38                  | 195                               | 500                             |
| ≥150              | ≥38                  | 210                               | 550                             |
| <b>Women</b>      |                      |                                   |                                 |
| 110-129           | ≥38                  | 180                               | 450                             |
| 130-149           | ≥38                  | 190                               | 450                             |
| 150-174           | ≥38                  | 190                               | 500                             |
| ≥175              | ≥38                  | 200                               | 550                             |

collected during the RBCP procedure according to donor weight and hematocrit. Plasma collected with the Autopheresis-C or PCS-2 is collected at a 1:16 AC:ACWB ratio, as is the plasma of the RBCP.

Apheresis FFP is a unique component and differs substantially from WBD FFP (Table 41-7). There is more absolute plasma per milliliter of anticoagulated plasma (90% in apheresis FFP vs 80% in WBD FFP), much less glucose, and only one-half to two-thirds of the citrate. A truly transfusable dose can be obtained that markedly affects the clotting factor status of an adult patient depending on patient size and the severity of the clotting deficiency. This component meets the criteria for a source of FFP espoused at the 1984 National Institutes of Health consensus conference on the use of plasma.<sup>27</sup> The number of residual platelets and white cells in apheresis FFP also is less than in WBD FFP. This may be important in reducing the recipient reactions to plasma transfusion caused by the release of cytokines or leukocyte fragments by passenger leukocytes into the plasma.<sup>23,24</sup>

Apheresis cryoprecipitate can be derived by means of cryoprecipitation of apheresis FFP. This product was not recognized by the FDA and has generally been abandoned. The Oklahoma Blood Institute previously produced apheresis cryoprecipitate from a volume of absolute plasma equivalent to 3 units of WBD FFP. The apheresis cryoprecipitate had a volume of approximately 100 mL (wet cryo) and did not require pooling or dilution but could be simply thawed and infused. This is in contrast to WBD cryoprecipitate, which is prepared as a dry cryo (volume, 15 to 20 mL) and requires dilution and pooling before administration.<sup>11</sup> The plasma remaining after removal of cryoprecipitate had a volume of approximately 400 mL and was used as replacement fluid for plasma exchange in the care of patients with thrombotic thrombocytopenic purpura (TTP).

Granulocyte donations are relatively infrequent because of the development of better antibiotics to prevent and manage the infections associated with granulocytopenia and because of the availability of cytokines such as GM-CSF and G-CSF, which help reduce the duration of the granulocytopenic period in patients with marrow failure. When granulocytes are needed, as in the management of neutropenia with bacterial or fungal sepsis not

**Table 41-7.** Comparison of Plasma Collected by Apheresis and Whole Blood Donation

|                                         | Collection Technique |          |                 |            |                         |
|-----------------------------------------|----------------------|----------|-----------------|------------|-------------------------|
|                                         | MCS+ LN8150          | ALYX     | PCS-2/Auto-C    | Concurrent | WBD FFP                 |
| Total volume (mL)                       | 450-550              | 450-550  | 500-800         | 250-450    | 200-250                 |
| Absolute plasma (%)                     | 90                   | 87       | 90              | 80-87      | 80                      |
| AC (% citrate)                          | CP2D (3%)            | CPD (3%) | Na Citrate (4%) | ACD (3%)   | CPDA-1, CPD, CP2D, (3%) |
| AC:ACWB ratio                           | 1:16                 | 1:12     | 1:16            | 1:8-1:12   | 1:8                     |
| Grams of citrate (per 100 mL of plasma) | 0.3                  | 0.4      | 0.4             | 0.6-0.4    | 0.6                     |

WBD = whole blood derived; FFP = fresh frozen plasma; AC = anticoagulant; CP2D = citrate-phosphate-dextrose-dextrose; ACD = acid-citrate-dextrose; CPDA-1 = citrate-phosphate-dextrose-adenine; CPD = citrate-phosphate-dextrose.

responding to antibiotics, collection of granulocytes poses problems for the donor and the patient. To collect an adequate number of granulocytes (see Chapter 15), the donor must be stimulated before donation with steroids and G-CSF to increase the circulating pool of granulocytes. Then hydroxyethyl starch (HES), available in low- and high-molecular-weight forms, must be infused during collection to increase red cell sedimentation and to facilitate separation and collection of granulocytes.<sup>28,29</sup> The use of G-CSF, steroids, and HES (pharmacomanipulation of the donor) can cause problems such as musculoskeletal pain, weight gain, fluid overload, allergic reaction to the starch, and reactivation of peptic ulcer disease by the steroids. However, only rarely are these problems severe.

HPCs can be collected with a variety of cell separators, but the Spectra, CS3000, and AS-104 are the most commonly used cell separators for HPC collection in the United States.<sup>30-33</sup> Circulating HPCs are collected by harvesting the buffy-coat-rich portion of the blood and specifically extracting the mononuclear-cell-rich portion of the buffy coat, which contains the CD34-positive progenitor or stem cells.<sup>33</sup> The number of CD34+ cells needed to repopulate the marrow varies from 4 to  $10 \times 10^6$ /kg of body weight. The HPCs usually are cryopreserved in autologous plasma with a 10% final concentration of dimethyl sulfoxide and are frozen in a controlled-rate freezer and stored at temperatures colder than  $-135^\circ\text{C}$ .

Collection of autologous HPCs by apheresis declined 26.1% between 2001 and 2004. Allogeneic HPC collections by apheresis decreased by 21.7% in the same period. Probable changes responsible for this decrease in total HPC collections by apheresis include: 1) marrow transplant protocols are no longer recommended for treatment of breast cancer patients, 2) new drugs such as rituximab and imatinib are used as first-line treatment rather than HPCs collected by apheresis, 3) newer nonmyeloablative protocols involve less processing and thus fewer collections, and 4) collection efficiency is increased by improved stem cell mobilization regimens. However, apheresis collection of allogeneic HPCs stimulated with G-CSF or GM-CSF as a replacement for classic allogeneic marrow HPC collection for hematopoietic reconstitution (see Chapter 33) is increasing in frequency. This may account for the rebound of allogeneic HPC apheresis collections in 2006, by an increase of 25.2%.<sup>4</sup> Autologous HPC apheresis collections also increased in 2006 — by 24.9%.<sup>4</sup>

### Adverse Effects on Donors and Recipients

Donor apheresis procedures are as safe or safer than donation of whole blood because of the ability to return fluids to donors and because the longer collection times allow better fluid equilibration and a lower risk of hypovolemia.<sup>34,35</sup> Hematomas with the use of single venous access devices (MCS+, Amicus, Trima) are no more frequent than in donation of whole blood. The major “expected” adverse consequence is related to the effects of the citrate anticoagulant returned to the donor. Donors of lower body weight are more likely to experience citrate symptoms. Almost all plateletpheresis donors and 2RBC donors experience some citrate effects

(circumoral paresthesia, buzzing sensation, and nasal stuffiness). These effects are not worrisome but they should trigger a slowing of the procedure before more serious toxic effects occur, such as muscle tightening, chills, nausea, vomiting, or tetany. Administering oral antacids, warming donors, and slowing the flow rate reduce the citrate effects. Calcium gluconate (10 mL of 10% solution given slowly intravenously) rarely is needed.<sup>36,37</sup> A single plateletpheresis procedure can remove 25% to 50% of circulating platelets, but the release of platelets that normally are sequestered in the spleen almost always prevents significant thrombocytopenia in the donor. In frequent plateletpheresis procedures, it is not uncommon for the donor’s platelet count to decrease and then rebound after 1 to 2 weeks.

Adverse effects in recipients of plateletpheresis products are equal to or less than the reactions that occur with administration of whole-blood-derived platelet products. The use of leukocyte reduction with almost all plateletpheresis products has markedly reduced but not eliminated transfusion-associated febrile reactions and appears to be reducing the incidence of transfusion-associated GVHD and posttransfusion purpura.<sup>38</sup> Transfusion-related acute lung injury (TRALI) is now recognized as one of the greatest noninfectious risks of transfusion. The greatest transfusion-associated infectious disease risk today is bacterial contamination and occurs almost exclusively in platelets. Because plateletpheresis products are from a single donor and a single collection event, the risk of bacterial contamination may be less with plateletpheresis products than with platelets from several whole-blood donations. However, bacterial contamination data from Europe showed no difference between pooled buffy coat whole-blood-derived platelets and apheresis platelet concentrates.<sup>39</sup>

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## Therapeutic Apheresis Overview

Therapeutic apheresis has been classified traditionally as therapeutic cytapheeresis or TPE, but must now include plasma immunoadsorption and cellular adsorption. Strictly speaking, TPE implies removal of the patient’s plasma with replacement of a crystalloid or colloid solution to maintain a normovolemic state in the patient. Plasmapheresis generally implies removal of an aliquot of plasma without fluid replacement. Because these procedures are discussed completely in Chapters 42 and 43, only basic principles are mentioned here.

Critical factors involved in therapeutic apheresis procedures include the following considerations:

- 1. What is to be removed and how best to remove it.** What is to be removed from the patient depends on the diagnosis. How it is to be removed depends on the equipment available. For patients with disease mediated by a known plasma factor such as an autoantibody, immune complex, a protein-bound drug or toxin, and a high cholesterol or triglyceride level, it is clear what must be removed.

- 2. How much is to be removed with each procedure.** How much plasma is to be removed depends on the plasma factor involved. The removal of one plasma volume results in a net

overall exchange of 62% of the patient's plasma volume because of progressive dilution by replacement solutions to maintain normovolemia during the procedure.<sup>40</sup> This is true whether plasma or red cells are being removed (Table 41-8). The most effective TPEs remove 1.0 to 1.5 plasma volumes (62% to 78% overall exchange). If the plasma factor is an IgG antibody, only about 45% to 50% of the total body IgG is within the intravascular compartment, whereas the remainder is in the extravascular spaces. Because equilibration is rapid, it is difficult to reduce IgG antibodies with only one TPE; daily TPEs are necessary. Conversely, approximately 90% to 92% of IgM antibodies reside in the intravascular compartment and are more easily removed with one large-volume TPE. Some institutions limit TPE to 1.0 to 1.5 calculated plasma volumes per procedure in most instances because of increased efficiency and a lower rate of complications than with larger-volume exchanges (2.0 volumes or greater).

A simple method for estimating the blood volume of adults is shown in Table 41-9. One of the authors (ROG) developed this modified rule of fives to quickly estimate blood and plasma volumes. It agrees with results of recent blood volume studies within  $\pm 15\%$ .<sup>41,42</sup> Plasma volume is calculated by means of multiplying the obverse of the hematocrit (1.0 minus the decimal equivalent of the hematocrit) by the estimated blood volume.

**3. How often the procedure should be performed.** How often a therapeutic apheresis procedure should be performed depends on how ill the patient is and the rate of regeneration or redistribution of the substance being removed. Removal of IgG antibodies generally requires daily TPE because of regeneration and redistribution from the extravascular to the intravascular space.

Therapeutic plasma exchange occasionally must be performed every 12 hours to save a patient with florid liver failure awaiting a liver transplant.

**4. How many procedures should be performed.** The end-point of therapeutic apheresis (TPE or therapeutic cytapheeresis) with regard to the number of procedures needed depends on the patient's clinical response and specific laboratory measurements. In disorders mediated by antibodies, only a limited number of procedures usually are needed. This is because TPE is generally a treatment that "buys time" during the wait for other therapeutic measures, such as immunosuppression, to take effect. Recurrence or exacerbation of some diseases, such as TTP or inflammatory neuropathy, frequently necessitates extending the therapeutic apheresis time frame to achieve durable remissions.

**5. What the replacement solution should be, if any.** Replacement solutions can be crystalloid or colloid (Table 41-10). Although they are less expensive, crystalloid solutions are less useful for large-volume daily plasma exchanges because of the rapid onset of hypoproteinemia and dilutional coagulopathy. In addition, the volume of crystalloid needed is at least two to three times the plasma removed because of the rapid movement of the crystalloid solution into the extravascular space. The most commonly used replacement solution in TPE is 5% albumin, a true natural colloid, which is slightly hyperoncotic. This form of albumin is stripped of its calcium and acts like a calcium sponge by binding calcium ions. It is a safe product, never having been associated with transmission of any known viral infection (HAV, HBV, HCV, or HIV). Because it is slightly hyperoncotic, albumin can be diluted with saline solution to 4.0% to 4.5% for plasma exchange. The other natural colloid is FFP, which

**Table 41-8.** Plasma Volumes Exchanged: Fraction Removed and Remaining

| Plasma Volume Removed | Fraction Removed (%) | Fraction Remaining (%) |
|-----------------------|----------------------|------------------------|
| 0.5                   | 40                   | 60                     |
| 1.0                   | 62                   | 38                     |
| 1.5                   | 78                   | 22                     |
| 2.0                   | 85                   | 15                     |
| 2.5                   | 91                   | 9                      |
| 3.0                   | 94                   | 6                      |

**Table 41-9.** Estimating Blood Volume: Modified Rule of Fives

| Gender | Body Mass and Build |      |        |          |
|--------|---------------------|------|--------|----------|
|        | Fat                 | Thin | Normal | Muscular |
| Men    | 60                  | 65   | 70     | 75       |
| Women  | 55                  | 60   | 65     | 70       |

Values are in milliliters of whole blood per kilogram of body weight.

**Table 41-10.** Replacement Solutions for Plasma Exchange

| Solution                         | Comment                                                                                                                |
|----------------------------------|------------------------------------------------------------------------------------------------------------------------|
| <b>Crystalloid Solutions</b>     |                                                                                                                        |
| Saline 0.9%                      | Least expensive, high Na and Cl                                                                                        |
| Ringer solution                  | Inexpensive, less Na and Cl                                                                                            |
| Lactated Ringer's solution       | Inexpensive, less Na and Cl                                                                                            |
| Balanced electrolyte solution    | Most physiologic but more expensive                                                                                    |
| <b>Colloids (Natural)</b>        |                                                                                                                        |
| Albumin 5%                       | Safe, hyperoncotic, and expensive                                                                                      |
| Plasma protein fraction 5%       | Not available in United States                                                                                         |
| Fresh frozen plasma (FFP)        | Less safe, isooncotic, contains clotting factors                                                                       |
| Donor retested FFP               | Slightly safer than FFP, more costly than FFP                                                                          |
| Solvent/detergent-treated plasma | Not available in United States, risk of non-lipid-enveloped virus transmission, pooled — includes Octaplas and Uniplas |
| <b>Colloids (Artificial)</b>     |                                                                                                                        |
| Hydroxethyl starch (high MW)     | 450,000 average MW                                                                                                     |
| Hydroxethyl starch (low MW)      | 150,000 average MW                                                                                                     |
| Dextran                          | Infrequently used                                                                                                      |
| Modified fluid gelatin           | Not in United States, used in Europe                                                                                   |

MW = molecular weight.

can be derived from whole blood or apheresis donation. As a byproduct of the RBCP procedure, apheresis plasma contains only half of the citrate of WBD FFP and is 90% absolute plasma vs 80% for WBD FFP. This is the replacement fluid of choice for patients with fulminant liver failure who require plasma exchange to save their lives.<sup>43</sup> However, because of an increased incidence of allergic reactions and slightly increased risk of transmission of infectious disease, FFP is generally used only when clotting factors or other proteins must be replaced or in the management of TTP, in which studies have established it as the replacement fluid of choice.<sup>44</sup> The issue of whether cryoprecipitate-reduced plasma, solvent/detergent-treated (SD) plasma, or unaltered FFP is best in the management of TTP is still not resolved. Artificial colloid solutions (HES or dextran) are used relatively infrequently in the United States and offer no medical advantage over 5% albumin. Depletion of immunoglobulins when using nonimmunoglobulin replacement solutions can put the patient at risk of pneumonia if the IgG levels drop too low. Plasma or IVIG can be given to prevent iatrogenic hypogammaglobulinemia from occurring. Two new SD plasma products are available (Octaplas) or in development (Uniplas) both of which contain normal levels of ADAMTS13 and Factor H, which are beneficial in treating TTP and atypical hemolytic uremic syndrome, respectively.<sup>45</sup> In addition, the use of Octaplas has resulted in a lower number of allergic and citrate reactions than the use of cryosupernatant plasma in acute TTP.<sup>46</sup> SD plasma is currently not available in the United States. Solvent/detergent treatment does not inactivate viruses that do not have a lipid envelope, such as hepatitis A or human parvovirus B19. SD plasma is a pooled product and has normal amounts of clotting factors but is deficient in some of the anticoagulating factors (protein C, protein S, and antithrombin) and plasminogen. Another plasma product with reduced infectious disease risk is donor retested plasma, which is rarely used in the United States. The donor is retested at least 110 days after the initial donation. The unit is not transfused until the retest has been performed and the results are negative for all viral markers tested. The development of pathogen-inactivated plasma will further reduce the risk of transmission of infectious agents but will not prevent the risk of prion transmission believed to be the etiologic agent for variant Creutzfeldt-Jakob disease.

**6. What equipment should be used.** The type of equipment used depends on the particular needs of the therapeutic apheresis service. The newest of the therapeutic devices in the United States is the Caridian Optia (see Table 41-1). Each of the different manufacturers' equipment has certain advantages. Most TPE procedures are performed with centrifugal-based machines. Add-on devices such as staphylococcal protein A immunoadsorption columns can be used *ex vivo* to remove IgG selectively and then to return the immunoadsorbed plasma to the patient.<sup>47</sup> Another special type of therapeutic apheresis, called *photopheresis*, is used to manage a hematologic malignant disease with cutaneous lesions called *cutaneous T-cell lymphoma* or *Sézary syndrome*. Photopheresis is the use of extracorporeal photochemotherapy to modify circulating malignant T cells. These

ultraviolet irradiated and altered T cells in some way modulate an antitumor effect when reinfused.<sup>48,49</sup> The new model of the Therakos Photopheresis System (UVAR-XTS) operates with a form of psoralen (methoxsalen) that is injected into the product collected with the device. This technique reduces the risks of systemic exposure to psoralen in the patient, which occurred in the original Therakos Photopheresis System (no longer available or supported).

**7. What type of vascular access should be used.** Vascular access can be through peripheral veins, central veins, or both. If many TPEs are planned, central venous catheters usually are necessary. Double- or triple-lumen dialysis catheters are ideal for central venous access. Strict catheter care is critical to prevent clotting of the catheter and infections at the catheter site. Flushing the catheter with a heparinized solution immediately after exchange is critical to prevent clotting. If catheter clotting should occur, injection of plasminogen-activating drugs into the clotted lumen frequently lyses the clot and restores patency. Placement of central venous catheters is critical so that the catheter tip is not in the heart (right atrium) but in the superior vena cava. Cardiac irritability with premature ventricular beats can occur if the catheter is in the right atrium. The irritation is caused directly by the catheter or indirectly by return of fluids that are cold, hypocalcemic, hypokalemic, or high in citrate ion. A chest radiograph should be obtained after placement of a subclavian central venous catheter, not only to locate the tip of the catheter within the superior vena cava but also to ensure that the catheter did not perforate the subclavian vein and cause a hemothorax or pneumothorax. For some patients, surgical placement of an arteriovenous shunt, such as an expanded polytetrafluoroethylene (Gore-Tex) graft tunneled under the anterior thigh to connect the femoral artery and vein, may be necessary for long-term TPE.

**8. What anticoagulant should be used and how much.** The use of anticoagulants in therapeutic apheresis usually is limited to citrate anticoagulants (ACD-A, ACD-B, or sodium citrate), heparin, or both. Acid-citrate-dextrose A (ACD-A) is a 3% citrate anticoagulant and ACD-B a 2% citrate anticoagulant. Sodium citrate is available as a 4% citrate anticoagulant. The advantage of citrate as an anticoagulant is that any amount returned to the patient during the red cell return in a TPE procedure is usually a small amount and is metabolized rapidly unless the patient is in severe liver failure. Citrate produces only local anticoagulation in the collected blood and never produces systemic anticoagulation. However, this can be a disadvantage when flow is low, because catheter clotting can occur. If the flow rate is low and heparin is not contraindicated, heparin can be used alone or in combination with citrate. Heparin may enhance systemic anticoagulation more than is expected because of the additional effect of dilution of clotting factors by the non-plasma replacement solutions. The half-life of heparin in the body is approximately 90 minutes. When heparin is used to anticoagulate blood in the draw line and is not used systemically, 17,500 U is added to 500 mL of saline solution (35 U of heparin per milliliter of saline solution). With

an AC:ACWB ratio of 1:8, this formula delivers 5 U of heparin per milliliter of whole blood collected. At an AC:WB ratio of 1:10, it delivers 3.9 U of heparin per milliliter of whole blood collected (3.5 U of heparin per milliliter of anticoagulated blood collected).

**9. Where the procedure should be performed, such as at the blood center or in the inpatient or outpatient areas of a hospital.** Therapeutic apheresis procedures should be performed only when emergency care is readily available. If the procedure is performed at the blood center on an outpatient basis, the blood center must have a knowledgeable physician available. The nursing staff performing the procedure must be trained in resuscitation care, and a crash cart with a cardiac monitor and defibrillator must be available. In the hospital, the same requirements apply for outpatient procedures. Critically ill patients, such as those with acute TTP, should be treated in an intensive care setting where emergency care is readily available.

**10. Whether the presence of a physician is needed throughout each procedure.** A physician must be available to the nurses for each procedure, but the severity of the patient's illness determines whether a physician is needed at the bedside for each therapeutic apheresis procedure. At the Oklahoma Blood Institute, a patient services department handles all therapeutic apheresis procedures, and all procedures are performed by knowledgeable registered nurses.

**11. What complications or adverse reactions are to be expected, such as thrombocytopenia, bleeding, thrombosis, arrhythmia, plasma-induced immune reactions.** Complications and adverse reactions can and do occur. Volume overload, venous access problems, bleeding, thrombosis, immunoglobulin depletion, citrate reactions, hypokalemia, hypocalcemia, hypotension, allergic reactions, infection, viral transmission from blood components, thrombocytopenia, anemia, TRALI, and death can occur. The mortality has been estimated at three deaths per 10,000 procedures.<sup>18</sup>

## Summary

Apheresis technology has rapidly advanced and is replacing some of the manual whole-blood collection techniques. Although more expensive and requiring personnel with greater skill levels to operate the equipment, the new techniques make it possible to collect two or more transfusion doses of blood components from a single donor, theoretically making safer and higher-quality blood components available from a smaller donor base and reducing the chances of error. The ability to use smaller needles and to return fluids will make procedures safer for donors as well. Therapeutic apheresis will continue to advance as new agents such as immunoabsorbents are combined with cell and plasma separation devices, whether centrifugal or membrane. The development of HPCs for marrow repopulation, especially when allogeneic donors are routinely stimulated with cytokines (eg, G-CSF), is signaling an even newer era of apheresis advancement. The new era of pathogen inactivation technology

and its application to replacement fluids will improve safety in the use of FFP derivatives for therapeutic apheresis.

## Disclaimer

The authors have disclosed no conflicts of interest.

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## Appendix 41-1. Internet Resources on Apheresis

AABB: [www.aabb.org](http://www.aabb.org)  
ASFA (American Society for Apheresis): [www.apheresis.org](http://www.apheresis.org)  
WAA (World Apheresis Association): [www.worldapheresis.org](http://www.worldapheresis.org)  
Fenwal: [www.fenwalinc.com](http://www.fenwalinc.com)  
Fresenius: [www.fresenius.com](http://www.fresenius.com)  
CaridianBCT: [www.caridianbct.com](http://www.caridianbct.com)  
Haemonetics: [www.haemonetics.com](http://www.haemonetics.com)  
Therakos: [www.therakos.com](http://www.therakos.com)

**Bruce C. McLeod**

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Therapeutic plasma exchange (TPE) has been compared to the practice of bloodletting to remove evil humors. The notion of therapeutic removal has changed little since medieval times; however, the concept of an evil humor has been discarded in favor of an evidence-based understanding that some blood cells and plasma components can be harmful. Three subtypes that can be distinguished among plasma macromolecules that are candidates for therapeutic removal are as follows: 1) antibodies that are troublesome because of their binding specificity—these are often autoantibodies; 2) molecules that confer abnormal physical properties, such as hyperviscosity or cold insolubility, on plasma and hence on the blood—these, too, are usually antibodies, although they may be bound in immune complexes; and 3) molecules such as low-density lipoproteins (LDLs) that have nonimmune toxicity. From a theoretic point of view, it is easier to envision a significant therapeutic effect when the molecule to be removed is relatively large and has a relatively long half-life in the circulation, with a correspondingly low rate of synthesis. In practice, the majority of successfully treated disorders are caused by pathogenic immunoglobulin G (IgG), which has these properties. It has been suggested from time to time that removal of complement or coagulation proteins, or inflammatory mediators derived from immunocytes, might contribute to a therapeutic effect. Such proposals have seemed unlikely to the author because the candidate molecules are rapidly synthesized and are relatively short lived, and in fact no therapeutic effect involving such molecules has been proven to date.

Therapeutic plasma exchange can be employed in a fourth way, not foreseen by medieval barbers or 18th century physicians, and that is to achieve relatively high levels of a normal plasma constituent that is deficient in patient plasma and is not available in a concentrated form for simple infusion.

### General Principles of Therapeutic Plasma Exchange

#### Mathematic Principles

Patients often compare TPE to an automobile oil change, and it is instructive to consider the factors that make this analogy inapt. A 100%-efficient 5-liter oil change can be accomplished in 10 minutes, because the engine need not operate during the procedure. In TPE, limitations are imposed on rate and efficiency by the need to keep the heart pumping and the bloodstream nearly full, so that only a small portion of the total blood volume can be outside the body at any time. Because TPE must proceed gradually, either continuously or in small increments, an ever-increasing proportion of the material being removed is not patient plasma but rather replacement fluid infused earlier in the procedure.

During this process, the level of an entirely intravascular substance that is absent in the replacement medium can be predicted by the formula:  $y_x = y_0 e^{-x}$ , in which  $y_0$  is the starting concentration of the substance,  $e$  is the base natural logarithm, and  $y_x$  is the concentration of the substance after  $x$  patient plasma volumes have been exchanged. If  $y_0$  is assigned a nominal value of 1.0, the function yields the smooth asymptotic middle curve plotted in Fig 42-1 for a continuous exchange. The flanking curves, describing small incremental discontinuous exchanges, are similar. This formula accurately forecasts the outcome of an exchange for macromolecules such as LDL and IgG that have a substantial extravascular reservoir, provided that equilibration between the intravascular and extravascular compartments is slow relative to the removal rate.<sup>2</sup>

Because the molecule targeted for removal by TPE is often an IgG antibody, which is approximately 50% extravascular,<sup>2</sup> and because removal of accessible (that is, intravascular) IgG becomes progressively less efficient during a TPE procedure, most practitioners limit an exchange to 1 to 1.5 times the patient's estimated plasma volume. An exchange of this magnitude will remove 60% to 75% of intravascular material while limiting side effects from depletion of normal plasma components. The intravascular IgG

### Section III

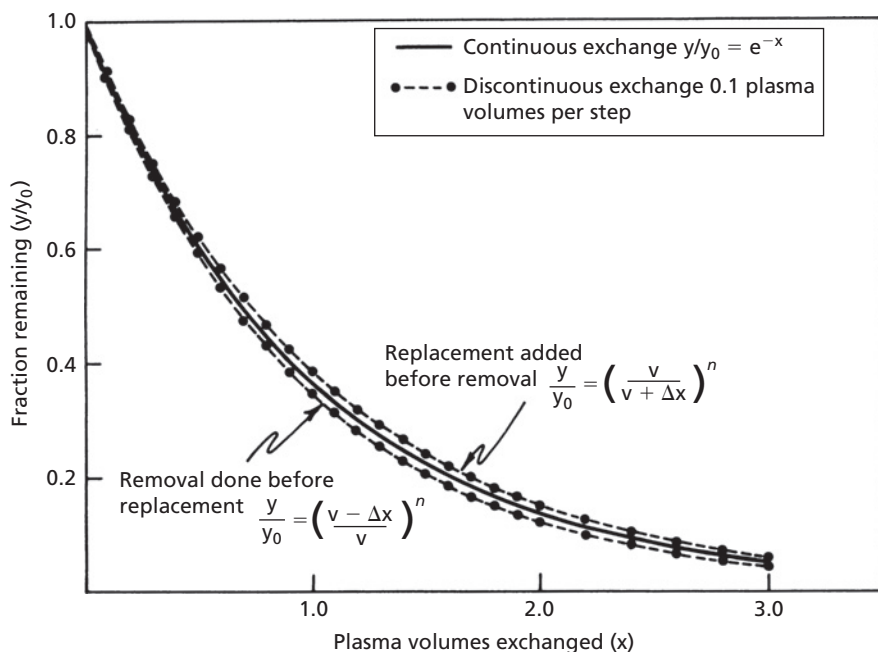


**Figure 42-1.** Calculated fraction of intravascular substance remaining during a plasma exchange, assuming no equilibration with extravascular material. (Used with permission from Chopek and McCullough.<sup>1</sup>)

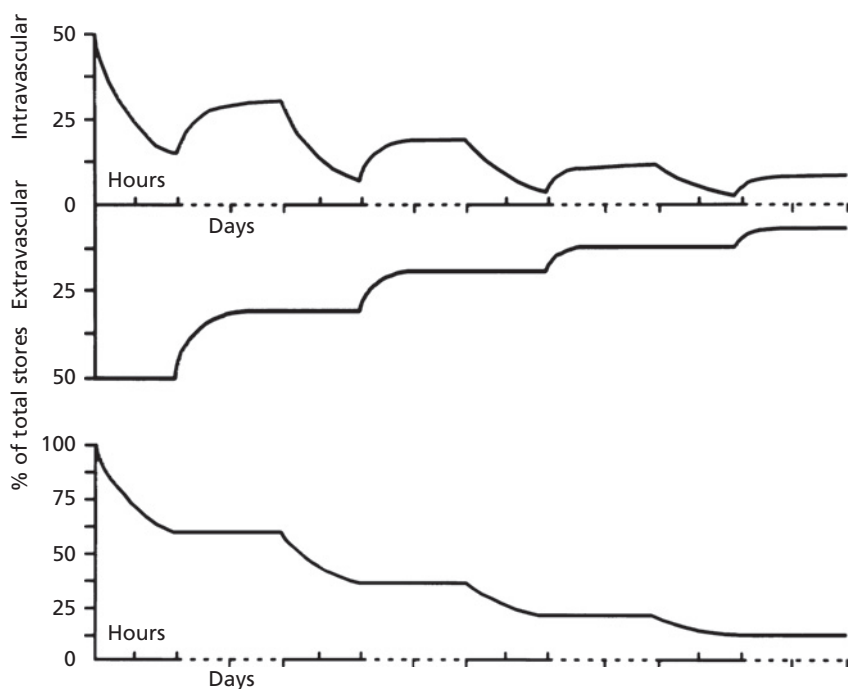


**Figure 42-2.** Computer-generated curve estimating amounts of intravascular and extravascular IgG (upper curves), and total IgG (lower curve) during a course of four one-plasma-volume therapeutic plasma exchanges with an IgG-free replacement medium. Published formulas were used for rates of removal during exchanges and reequilibration after exchanges. No correction was made for continuing synthesis.

level will rise during the ensuing 1 to 2 days by equilibration with extravascular sources, and further removal by a subsequent exchange can then be undertaken more efficiently. The effects of a series of TPEs on extravascular, intravascular, and total IgG are shown schematically in Fig 42-2.

Human serum albumin is the most common replacement fluid in TPE, for reasons that are given below. In such exchanges,

all plasma constituents are removed while only albumin is replaced; thus plasma component depletion in an individual exchange is almost completely nonselective.<sup>2</sup> However, because most other plasma constituents are synthesized much more rapidly than IgG, a series of such exchanges will result in a reasonably selective depletion of IgG over the course of treatment. This is shown in Fig 42-3.

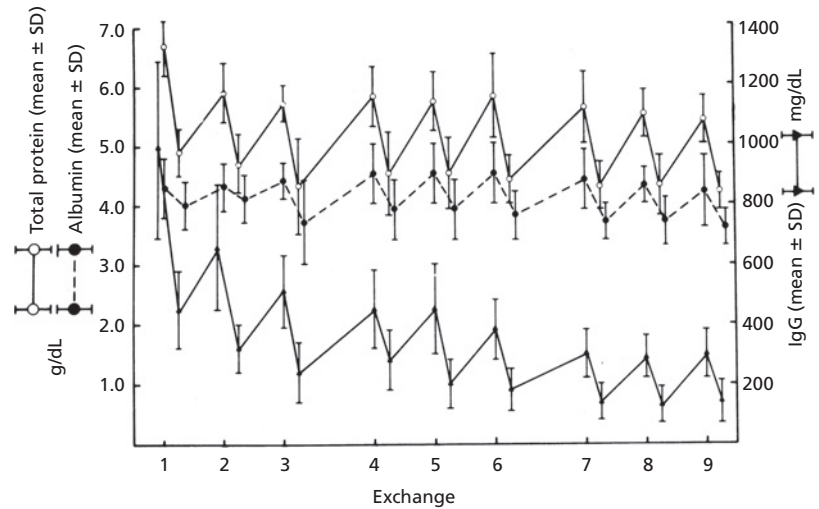


**Figure 42-3.** Total protein, albumin, and IgG levels before and after therapeutic plasma exchange with albumin/saline replacement. Exchanges were carried out three times per week for 3 weeks on seven patients. Points and ranges represent means and standard deviations. Note this disproportionate decrease in IgG levels. (Used with permission from McLeod et al.<sup>3</sup>)

### Regulation of IgG Metabolism

Because IgG removal is often the goal of TPE, it is worthwhile to consider certain aspects of IgG metabolism. The subclasses IgG1, IgG2, and IgG4 together constitute about 90% of total IgG. Their catabolic rates are proportional to total IgG level and their half-lives are therefore inversely proportional to concentration. This pattern has been taken to indicate the existence of a saturable receptor that protects IgG from catabolism. A receptor on endothelial cells having this property has been characterized, which appears to be identical to the FcRn receptor on neonatal intestinal epithelial cells that facilitates transport of intact maternal antibody in breast milk.<sup>4</sup>

It is difficult to measure the IgG synthetic rate in humans. Previous animal studies concerning levels of specific antibody were interpreted to show that the synthetic rate for IgG exhibits negative feedback, increasing when IgG or specific antibody levels or both are lower.<sup>5</sup> More recently, Junghans<sup>6</sup> has shown that “knock-out” mice genetically deficient for the FcRn receptor catabolize IgG quite rapidly and maintain very low IgG levels, but they have the same IgG synthetic rate as normal mice. This argues against negative feedback regulation of IgG synthesis and suggests that a reduction in antibody levels induced by TPE would not produce a meaningful “rebound” increase in IgG synthesis.

Intravenous immune globulin (IVIG) has been reported to be effective in some of the same antibody-mediated diseases as TPE. Yu and Lennon<sup>7</sup> recently proposed that exogenous immune globulin competes with endogenous IgG for FcRn receptors and thereby promotes accelerated catabolism of the latter, including any pathogenic antibodies. In this view, the beneficial effects of IVIG and TPE are essentially the same; both lead to lower levels of harmful antibodies. Therapeutic plasma exchange lowers levels quickly but may be followed by slower catabolism, whereas IVIG presumably has a slower onset of action but promotes rapid catabolism. This concept of the effect of IVIG could provide a

**Table 42-1.** Colloid Replacement Fluids for TPE

| Fluid                            | Advantages                                                           | Disadvantages                                                                                            |
|----------------------------------|----------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------|
| 5% Albumin                       | Virus inactivation<br>Ease of use<br>Reactions rare                  | High cost<br>Most proteins not replaced                                                                  |
| Single-donor plasma*             | All proteins replaced                                                | High cost<br>Inconvenient†<br>Citrate reactions<br>Urticaria<br>Viral infection risk                     |
| Solvent/detergent-treated plasma | All proteins partially replaced‡<br>Lipid-coated viruses inactivated | Very high cost<br>Inconvenient†<br>Citrate reactions<br>Urticaria<br>Pooled product<br>Unavailable in US |
| 6% Hetastarch                    | Low cost<br>Viral safety<br>Ease of use<br>Slow catabolism           | No proteins replaced<br>Hypotensive reactions<br>Dosage limit                                            |

\*Fresh Frozen Plasma or Plasma Cryoprecipitate Reduced.

†Must be thawed before use; must match patient ABO group.

‡Coagulation factors ≥80% of normal levels.

TPE = therapeutic plasma exchange; US = United States.

compelling rationale for sequential treatment of autoantibody diseases with TPE followed by IVIG.

### Replacement Fluids

Saline replacement alone can suffice when only 500 to 1000 mL of plasma is removed in a manual plasmapheresis, but colloid replacement must be given in a multiliter plasma exchange.

Available colloid replacement fluids are listed in Table 42-1, which also summarizes their relative advantages and disadvantages. For the majority of indications the standard replacement medium is 5% human serum albumin in normal saline; however, substitution of 25% to 50% of the total with saline has been shown to be well tolerated in certain patient groups. Although it is a pooled product, 5% albumin is preferred over plasma as a source of replacement colloid because 1) it can be pasteurized to inactivate all known blood-borne pathogens, 2) it can be given without regard to blood type, and 3) it does not require thawing or other preparation before use. Adverse reactions to albumin are rare.<sup>8</sup> Exchanges of plasma for albumin produce temporary deficiencies of other plasma proteins, such as coagulation factors; however, these are usually sub-clinical and levels are rapidly restored by ongoing synthesis and reequilibration.<sup>2</sup>

There are a few circumstances in which replacement of patient plasma with donor plasma seems prudent. Prominent among these is the treatment of thrombotic microangiopathies. Affected patients customarily receive exchanges with Fresh Frozen Plasma (FFP) in light of abundant evidence that patients with thrombotic thrombocytopenic purpura (TTP) respond better to plasma than to albumin. Cryoprecipitate-reduced plasma is also effective for this purpose.<sup>9</sup> Some plasma may also be given toward the end of an exchange to patients with preexisting thrombocytopenia or humoral coagulopathy, who are considered to be at increased risk for bleeding complications when the dilutional coagulopathy of an albumin exchange is superimposed, or to patients with ongoing blood loss regardless of pretreatment coagulation status. Plasma replacement carries a higher risk of urticarial and hypocalcemic reactions.<sup>10</sup>

One group reported infusing a solution of hydroxyethyl starch (HES), a less costly volume expander, in the early part of an exchange.<sup>11</sup> They reason that recommended dosage limitations for HES will not actually be exceeded, because much of the infused HES will be removed in exchange for albumin infused later. This group has seen successes, albeit with a higher incidence of side effects. HES is not recommended for patients with renal impairment, underlying coagulopathy, or a history of hypersensitivity to HES.

Selective Extraction of Plasma Components

Practitioners of TPE have long recognized the inherent wastefulness in removing and discarding all plasma components in order to deplete just one. Selective extraction of a pathogenic component from separated plasma, with recovery and reinfusion of normal constituents, has been considered an attractive goal. Several applications and methods for on-line separation have been explored,<sup>12</sup> but commercial availability and widespread acceptance have been slow to be realized.

Obstacles to commercially practical devices include the high cost and limited capacity of truly selective, biocompatible sorbents and the high extracorporeal volume associated with

sorbent modules having a depletion capacity equivalent to TPE. One approach is to use pairs of small, low-capacity sorbent modules with alternating flow cycles such that one module absorbs material from patient plasma while the absorbent capacity of the other is restored by elution of bound material. This approach to selective extraction lowers the volume and cost of sorbent modules but requires an additional instrument to manage the cycling-elution-regeneration process.<sup>12</sup> Selective extraction techniques and their applications are discussed in more detail in Chapter 41.

Indication and Treatment Intensity Categories

To assist practitioners in assessing the value of apheresis in specific circumstances, the American Society for Apheresis (ASFA) has described indication categories for therapeutic apheresis.<sup>13</sup> The definitions can be summarized as follows: Category I denotes diseases for which apheresis therapy is a standard first-line therapy, although it may not always be necessary; Category II denotes diseases in which apheresis is a valuable second-line therapy when first line measures fail or are poorly tolerated; Category III indicates uncertainty due to inadequate data or controversy due to conflicting reports; and Category IV is reserved for diseases in which there is negative data from controlled trials or anecdotal reports. The indication categories assigned by ASFA are provided in tabular form for the diseases in each of the following sections.

In addition, these tables include the author’s perceptions of the customary intensity (frequency and duration) of TPE therapy for most of the diseases. The four categories used are: aggressive (A), which implies daily TPE until remission or improvement; routine (R), which implies five to seven treatments every other day (or three times per week); prolonged (P), which implies one to three treatments per week for 3 to 8 weeks; and chronic (C), which implies one treatment every 1 to 4 weeks, continuing indefinitely. These intensity categories, which need not be mutually exclusive, are summarized in Table 42-2. Note that more than one category may be applicable to a given disease, or even a given patient, depending on extant clinical circumstances.

The references cited in the following discussions are not exhaustive. Readers seeking a more extensive bibliography may consult the chapters on TPE in a recent text devoted to apheresis<sup>2,12,14-16</sup> and articles in recent journal issues devoted to clinical applications of therapeutic apheresis.<sup>13,17-20,21</sup>

Table 42-2. Intensity of Treatment Categories for TPE

| Level          | Schedule         | Duration                   |
|----------------|------------------|----------------------------|
| Aggressive (A) | Daily            | 3 treatments to indefinite |
| Routine (R)    | 3 times a week   | 5-7 treatments             |
| Prolonged (P)  | 1-2 times a week | 3-8 weeks                  |
| Chronic (C)    | Every 1-4 weeks  | Indefinite                 |

TPE = therapeutic plasma exchange.

## Therapeutic Plasma Exchange in Neurologic Disorders

Immune processes, especially formation of circulating antibody to structures in the nervous system, have been implicated in several neurologic diseases, and TPE has become an important therapy for some of them. The diseases to be considered in this section appear in Table 42-3 along with their respective indications and intensity categories.

### Guillain-Barré Syndrome

Guillain-Barré syndrome (GBS) affects the peripheral nervous system. It is the most common cause of acute paralysis with areflexia in the developed world, with an incidence of one to two cases per 100,000 population per year. A typical clinical course begins with symmetric distal paresthesias, which are followed by leg and arm weakness. Symptoms spread proximally and reach a peak of severity by 14 to 30 days after onset. About one-fourth of patients with GBS have mild illness and remain ambulatory throughout. The remainder are disabled by paralysis and may have oropharyngeal and respiratory weakness as well. One-fourth will require assisted ventilation at some point, and the worst cases are marked by quadriplegia, ophthalmoplegia, and prolonged ventilator dependence. A nerve conduction defect resulting from demyelination is found in most cases, but there are variants in which axonal damage is evident in motor fibers or both motor and sensory fibers. In another variant (Miller Fisher syndrome) symptoms are limited to ophthalmoplegia, ataxia, and areflexia. The spinal fluid usually contains few cells and only a moderately elevated concentration of protein. A conduction block indicating demyelination is the usual finding in electrophysiologic studies, although inexcitability may also be seen in the axonal variants.<sup>22</sup>

Demyelination is believed to be caused by circulating myelin antibodies. Early experiments showed that sera from patients with GBS produced demyelination in animals. Later studies have found antibodies to myelin glycolipids, such as GM1, GM1b, GD1a, and GD1b, in the sera of many patients. Antibodies to GQ1b and GT1a are relatively specific for the Miller Fisher variant. Guillain-Barré syndrome is often associated with a history of recent infection with *Campylobacter jejuni*, cytomegalovirus, Epstein-Barr virus, *Mycoplasma pneumoniae*, or other organisms. It has been postulated that antibodies are formed in response to a strain-specific lipopolysaccharide that is antigenically similar to one of the myelin gangliosides; eg, GM1 after *C. jejuni* infection.<sup>22</sup> Epidemic Chinese acute motor neuropathy is an illness of rural Chinese children that has epidemiologic characteristics of an infectious disease. It is clinically similar to GBS and is also associated with evidence of *C. jejuni* infection and GM1 antibodies.<sup>23</sup>

Spontaneous recovery is the usual outcome of GBS and may be associated with the decline in antibody levels expected after recovery from infection. Patients with mild illness require no treatment, but more severely affected patients need careful observation so that appropriate supportive therapies, such as mechanical ventilation, can be implemented when needed. Neither oral nor pulse intravenous steroids are helpful in GBS; however, large randomized controlled trials have documented that TPE can shorten recovery time and reduce disability.<sup>24-27</sup>

The North American trial enrolled 245 disabled patients, 142 of whom received TPE.<sup>24</sup> At 4 weeks, 59% of treated patients vs 39% of controls had improved by one grade in a clinical grading scale devised for the study. Mean improvements were 1.1 and 0.4 grades, respectively. The median time to improve one grade was 19 days in treated patients vs 40 days in controls, while median

**Table 42-3.** Indication and Intensity of Treatment Categories for TPE in Neurologic Disorders

| Disorders                                         | Antibody Specificities          | ASFA Indication Category* | Intensity Category† |
|---------------------------------------------------|---------------------------------|---------------------------|---------------------|
| Guillain-Barré syndrome                           | Peripheral nerve myelin         | I                         | R                   |
| Chronic inflammatory demyelinating polyneuropathy | Peripheral nerve myelin         | I                         | P                   |
| Peripheral neuropathy with monoclonal gammopathy  | Myelin-associated glycoprotein  | I, II                     | P                   |
| Myasthenia gravis                                 | AChR                            | I                         | A, R, P, C          |
| Lambert-Eaton myasthenic syndrome                 | Voltage-gated calcium channel   | II                        | R                   |
| Neuromyotonia and limbic encephalitis             | Voltage-gated potassium channel | NR                        | R, C                |
| Stiff-person syndrome                             | GAD-65                          | III                       | R                   |
| Paraneoplastic neurologic syndromes               | Various                         | III                       | R                   |
| Rasmussen encephalitis                            | Glu R3                          | II                        | R                   |
| Sydenham chorea                                   | Unknown                         | I                         | R                   |
| PANDAS                                            | Unknown                         | I                         | R                   |
| Multiple sclerosis                                | Unknown                         | III                       | R                   |

\*I = standard first-line therapy; II = second-line therapy; III = controversial; IV = no efficacy.

†A = aggressive; R = routine; P = prolonged; C = chronic (see Table 42-2).

TPE = therapeutic plasma exchange; ASFA = American Society for Apheresis; AChR = acetylcholine receptor molecule; NR = not rated; GAD-65 = 65-kD isoform of glutamic acid decarboxylase; Glu R3 = glutamate receptor; PANDAS = pediatric autoimmune neuropsychiatric disorders associated with streptococcal (infection).

times to walk unassisted were 53 and 85 days, respectively. In the subgroup of ventilated patients, the median times to weaning were 24 and 48 days, respectively, and the median times to walk unassisted were 97 vs 169 days. A similar shortening of recovery time in severely affected patients was shown in the French Cooperative Group trial involving 220 patients.<sup>25</sup> In another study of 556 patients, this group showed benefit from TPE in mildly affected patients as well.<sup>26</sup> A typical treatment schedule in these trials consisted of five to six exchanges of 1.0 to 1.5 plasma volumes over 7 to 14 days. Patients with GBS may need careful monitoring, perhaps in an intensive care unit, because they may have autonomic neuropathy and are more prone to hemodynamic instability during TPE.

Later studies have shown that IVIG is also beneficial in GBS. A large multicenter trial compared IVIG, TPE, and TPE followed by IVIG, with 121 to 130 patients in each treatment group.<sup>28</sup> Mean disability grades at 4 weeks improved by 0.8, 0.9, and 1.1 respectively, while the median times to walk unassisted were 51, 49, and 40 days. The trends favored TPE plus IVIG, although none of the differences was statistically significant. Some authorities now favor IVIG for initial treatment, citing its relative simplicity, wider availability, and lower incidence of adverse effects.<sup>22</sup>

### Chronic Inflammatory Demyelinating Polyneuropathy

Chronic inflammatory demyelinating polyneuropathy (CIDP) is an acquired neuropathy that may follow either a continuously progressive or an intermittent, relapsing course. Both weakness and sensory loss are usually present, and both distal and proximal sites may be affected. Proximal weakness can help to differentiate CIDP from other chronic neuropathies, while progression for more than 2 months helps to distinguish it from GBS. Nerve conduction studies should suggest demyelination, which may be apparent in nerve biopsy tissue if this is obtained. Patchy inflammatory infiltrates may be seen in nerve root biopsies. The cerebrospinal fluid usually has a moderately elevated protein concentration and a cell count less than  $10/\mu\text{L}$ .<sup>29</sup> If certain exclusionary criteria are met, the diagnosis of CIDP is deemed "typical" or "atypical" on the basis of clinical findings and "definite," "probable," or "possible" based on electrophysiologic studies in one recent classification scheme.<sup>30</sup> Treatment is recommended for all groups, and the results of treatment are similar in all.<sup>31</sup> Although it is usually idiopathic, CIDP may also occur in the context of an associated condition, such as inflammatory bowel disease, chronic active hepatitis, connective tissue disease, Hodgkin disease, human immunodeficiency virus (HIV) infection, or monoclonal gammopathy.<sup>29</sup>

The precise cause of CIDP remains unknown; however, the disease associations, the clinical similarities to GBS, and the histopathology suggest an immune process. The presence of a monoclonal protein in some cases points to an antibody-mediated disorder, as does the finding that an animal model of CIDP (experimental allergic neuritis) can be passively transferred with serum. Recent studies have demonstrated antibody to myelin protein antigens such as P0, P2, and PMP22 in the sera

of some patients with CIDP, although a definite cause-and-effect relationship has not been established.<sup>32</sup>

Most patients with CIDP will respond to moderately high doses of glucocorticoids.<sup>29</sup> Early trials of TPE were conducted in patients with CIDP who had failed to improve with steroids or were unable to tolerate them. A double-blind, sham-controlled crossover trial reported by Dyck et al<sup>33</sup> showed improvement in five of 15 patients treated with TPE for 3 weeks; after crossover a similar proportion responded in the sham group. However, many patients worsened after TPE was stopped. A similar study in 18 previously untreated patients was reported by Hahn et al.<sup>34</sup> Of the 15 patients who completed the trial, 12 improved during the TPE portion. These patients took prednisone for 6 months after completing TPE and, following recovery from a brief relapse when TPE was stopped, many maintained good function.

IVIG has also been investigated in CIDP. Hahn et al<sup>35</sup> published a double-blind, placebo-controlled crossover trial in 30 patients. Study patients were permitted to take prednisone at stable low doses. Overall 63% (19 patients) responded to IVIG, while 17% (five patients) improved on placebo. Two studies have compared IVIG to TPE. In a retrospective study by Choudhary and Hughes,<sup>36</sup> 21 of 33 patients (64%) improved after TPE treatment, while a response to IVIG was seen in 14 of 21 patients (67%) treated at the same institution. A prospective observer-blinded crossover study of 20 patients at the Mayo Clinic suggested that both therapies were able to produce rapid, statistically significant improvement; the authors concluded that either was appropriate as a primary treatment.<sup>37</sup> Some authorities recommend using IVIG first, based on its relative simplicity, wide availability, and lower incidence of adverse effects.<sup>30</sup> The TPE protocols used in CIDP have tended to specify relatively prolonged treatment.<sup>21(p121)</sup> A proposed schedule is three one-plasma-volume exchanges each week for 2 weeks, followed by two exchanges each week for another 4 weeks.<sup>33</sup>

### Peripheral Neuropathy and Monoclonal Gammopathy

The background incidence of a circulating monoclonal immunoglobulin is about 1% in adults over 50 and rises to about 3% in adults over 70, whereas about 10% of patients with polyneuropathy have such a protein.<sup>38</sup> Thus, principles of epidemiology suggest a causal relationship in at least some cases. This idea is supported by studies showing that myelin antibody activity is expressed by the monoclonal proteins of many patients with neuropathy. Specificity for a carbohydrate epitope on myelin-associated glycoprotein (MAG) can be identified in a majority of IgM-associated neuropathies. The same epitope is found on myelin glycoprotein P<sub>0</sub> and on other gangliosides in nerve cell membranes. Injection of anti-MAG into experimental animals is known to produce demyelination. Additional patients have antibody activity against myelin sheath sulfatides, membrane-associated chondroitin sulfate C moieties, or various myelin gangliosides.<sup>20,39</sup>

Most clinical features of neuropathies associated with a monoclonal gammopathy are similar to CIDP<sup>29</sup>; however, sensory

abnormalities tend to be more prominent and, while progression may be slower overall, it is also more relentless, so that instances of spontaneous improvement are uncommon. The prevalence of neuropathy is higher in patients with IgM paraproteins than in those with IgG or IgA except in osteosclerotic myeloma, where the prevalence of neuropathy with IgG or IgA proteins is quite high, sometimes as a part of the POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, monoclonal protein, skin changes). Nerve biopsies show demyelination, and immunofluorescence may demonstrate IgM and complement in IgM-associated cases.<sup>38,39</sup>

Patients with multiple myeloma or Waldenström's macroglobulinemia should be treated with appropriate chemotherapy and may experience improvement in the neuropathy as a result. However, many patients have neuropathy in addition to a monoclonal gammopathy of undetermined significance (MGUS) and are usually treated with immunosuppressive regimens similar to those used in CIDP.<sup>38</sup>

Therapeutic plasma exchange can be helpful in MGUS-associated neuropathy. In a sham-controlled trial in 39 patients, twice-weekly exchange led to improvement in disability scores, weakness scores, and electrodiagnostic parameters in the blinded portion of the trial. Scores also improved in sham-treated patients who received true TPE in an open follow-up study.<sup>40</sup> In this and other studies, a response was noted more frequently in patients with IgG and IgA paraproteins than in those with IgM.<sup>14,21(p146)</sup>

### Myasthenia Gravis

Myasthenia gravis is a disease of the neuromuscular junction that is characterized clinically by fatigability, with or without weakness in skeletal muscle. Ocular myasthenia, with diplopia and ptosis, may be the initial presentation, but other muscles are eventually affected in most patients. Involvement of muscles innervated by lower cranial nerves leads to the most serious symptoms, including dysphagia and respiratory insufficiency.<sup>41</sup>

About 85% of patients with myasthenia have circulating antibody to a portion of the  $\alpha$ -subunit of the acetylcholine (ACh) receptor molecule (AChR) on the motor end plate of muscle cells. Up to 41% of those lacking anti-AChR have circulating antibody to a muscle-specific kinase (MuSK) that is also found in the motor end plate. A few others have antibody to the muscle cytoplasmic proteins titin or RyR.<sup>41</sup>

There are two kinds of drug therapy for myasthenia. Acetylcholinesterase inhibitors, such as neostigmine and pyridostigmine, slow the degradation of ACh at the neuromuscular junction, enhancing its action on remaining receptors. Immunosuppressive drugs, such as prednisone and azathioprine, are also useful. These agents reduce damage to receptors through general antiinflammatory properties or decreased antibody synthesis or both.<sup>42</sup>

Surgical treatment is also employed. Malignant thymoma is sometimes associated with myasthenia in younger patients. Strength may improve in such patients after surgical resection of the thymus. This observation led to trials of thymectomy in younger patients without tumors, who may also experience disease remission.<sup>41,42</sup>

Because of the relationships between circulating antibody, pathology, and symptoms, TPE has seemed a very reasonable approach to treatment for myasthenia. Although a controlled trial has never been published, numerous open trials have suggested that TPE can lead to rapid symptomatic improvement in concert with lower levels of circulating AChR antibody. Therapeutic plasma exchange has also been effective in patients who test negative for antibody, suggesting that some pathogenic antibodies may not be detected by current assays.<sup>20,41</sup>

As a result of favorable experience, TPE is a widely accepted therapy for myasthenia; however, it is not recommended for all patients. It is instead reserved for those with severe disease and those who are intolerant of, or unresponsive to, other therapies. Patients whose breathing, swallowing or walking is inadequate are good candidates for the rapid improvement brought about by TPE, even as an initial treatment.<sup>42</sup> Therapeutic plasma exchange can also be useful to optimize muscle function before thymectomy or other surgery. An occasional patient will need regular TPE at 2- to 4-week intervals in addition to maintenance drug therapy for optimal function.

Myasthenia may respond to IVIG. No randomized trials have been conducted to prove efficacy; however, in a controlled study comparing IVIG to TPE in 87 patients, either three or five infusions of 0.4g/kg IVIG were equivalent to three TPE treatments. The median time to response was shorter for TPE-treated patients (9 days vs 15 days), but not significantly so ( $p = 0.14$ ).<sup>43</sup> A multicenter retrospective chart review found that ventilatory status at 2 weeks and functional status at 1 month were significantly better in patients treated with TPE.<sup>44</sup>

### Lambert-Eaton Myasthenic Syndrome

Lambert-Eaton myasthenic syndrome (LEMS) is also characterized by weakness and fatigue. Neither bulbar nor oculomotor symptoms are likely to be prominent in LEMS, but signs of dysautonomia, such as dry mucous membranes and orthostatic hypotension, are common. The syndrome is most often seen in patients with cancer, with almost half of cases being in patients with small cell lung cancer. Neuromuscular symptoms may precede any other sign of tumor.<sup>45</sup>

The pathophysiology of LEMS also involves the neuromuscular junction, but the defect is in the nerve cell ending instead of the muscle cell. Symptoms are caused by circulating antibody against "active zones" in the nerve terminus, which house the voltage-gated calcium channels that mediate electrical events in neuromuscular impulse transmission. These antibodies reduce the amount of ACh released during depolarization events, causing weakness in affected skeletal muscles as well as dysfunction in autonomic nerves.<sup>45</sup>

Curiously, cholinesterase inhibitors are not as effective in LEMS as they are in myasthenia gravis.<sup>46</sup> More useful are agents that prolong nerve action potentials by blocking voltage-gated potassium channels (VGKCs). These drugs may improve muscle strength in patients with LEMS, presumably by enhancing ACh release; 3,4-diaminopyridine is the most useful agent of this type.



Immunosuppressive drugs such as prednisone and azathioprine may also be beneficial in LEMS, and paraneoplastic cases may respond to specific antitumor therapy.<sup>45,46</sup>

Therapeutic plasma exchange has been helpful in LEMS. Responses are usually less dramatic than those seen in myasthenia gravis, perhaps suggesting that a damaged nerve ending needs more time to heal than a motor end plate. IVIG has also been reported to be active in both the short and long term.<sup>45,46</sup>

### Neuromyotonia and Limbic Encephalitis

Neuromyotonia is characterized by muscle cramps, involuntary contractions, fibrillations, and fasciculations. There is evidence to suggest that some cases are caused by the presence of antibodies to VGKC in nerve fibers. VGKC have a role in terminating action potentials, such that their blockage prolongs action potentials and enhances ACh release. Therapeutic plasma exchange has been followed by clinical improvement in individual cases.<sup>47</sup> VGKC antibodies have also been found in some patients with limbic encephalitis characterized by memory impairment, often with preservation of general intelligence. Therapeutic plasma exchange has led to improvement in individual non-paraneoplastic cases, in concert with diminution in antibody levels.<sup>48</sup>

### Stiff-Person Syndrome

Stiff-person syndrome (SPS), originally known as stiff-man syndrome, is characterized by progressive rigidity and/or spasms of trunk and proximal limb muscles. About 60% of patients have high titers or circulating antibody to the 65-kD isoform of glutamic acid decarboxylase (GAD-65), which is the rate-limiting enzyme in the synthesis of  $\gamma$ -amino butyric acid (GABA), the neurotransmitter at many inhibitory central nervous system (CNS) synapses. When present, anti-GAD-65 is believed to have a pathogenic role, even though neuronal GAD is a cytoplasmic enzyme. CNS histology is normal in SPS, suggesting that anti-GAD causes functional rather than structural changes. Pathogenesis is more uncertain in patients who lack antibody to GAD. About 5% of cases are associated with cancer, usually breast cancer. Instead of anti-GAD, most such patients have antibody to amphiphysine, a 128-kD presynaptic vesicle protein. A few have antibody to a postsynaptic protein called gephyrin that is associated with receptors for GABA and for glycine, another inhibitory neurotransmitter. Symptoms often respond to diazepam or other agents that increase CNS GABA levels. Immunomodulatory therapies such as prednisone, TPE, and IVIG have also seemed beneficial in individual cases, but no controlled trials of TPE have been performed. In paraneoplastic cases improvement may follow removal of the malignancy.<sup>21(p156),49</sup>

### Paraneoplastic Neurologic Syndromes

In addition to LEMS, several other neurologic syndromes associated with malignant tumors are characterized by circulating antibody to structures in the nervous system. Paraneoplastic encephalomyelitis includes seizures, mental changes, and cerebellar and autonomic dysfunction. It is often associated with

anti-Hu (also called ANNA-1), an antibody to a 38- to 40-kD antigen in the nuclei of neurons and of small cell lung cancer cells. Paraneoplastic cerebellar degeneration is characterized by ataxia, dysarthria, and down-beating nystagmus. It may be associated with ovarian, breast, and small cell lung cancers, as well as with Hodgkin disease. About 40% of these patients have an antibody to 34- and 62-kD antigens in Purkinje cells (anti-Yo). Paraneoplastic opsoclonus-myoclonus syndrome produces both vertical and horizontal dysrhythmic conjugated eye movements. It may occur in children with neuroblastoma and in adults with lung, breast, or other tumors. Cases seen with breast or gynecologic cancers may have circulating anti-Ri (also called ANNA-2), an antibody to 55- and 80-kD antigens in neuronal nuclei. Paraneoplastic SPS is characterized by stiffness and spasm in axial muscles. It is associated with antibody to amphiphysin, a 128-kD synaptic vesicle protein. Cancer-associated retinopathy produces photosensitivity and gradual vision loss. It is associated with anti-CAR, an antibody to antigens shared by retinal neurons and small cell lung cancer cells.<sup>50</sup>

Treatment of these syndromes is uniformly difficult. They seldom respond well to immunosuppressive drugs or to antitumor measures, even when these are otherwise effective. Therapeutic plasma exchange has been tried in these syndromes, usually with disappointing results.<sup>21(p142),50</sup>

### Nonneoplastic Disorders with Central Nervous System Antibodies

Rasmussen's encephalitis is a rare, acquired disorder that begins in childhood, often following a viral infection. Seizures are a prominent feature; however, unlike patients with idiopathic epilepsy, those with Rasmussen's encephalitis develop progressive, predominantly unilateral neurologic deficits including hemiparesis and mental retardation. The histopathology includes inflammation and atrophy of brain tissue, usually confined to one hemisphere.<sup>51</sup> Recent studies have revealed circulating IgG antibody to the Glu R3 receptor for the CNS neurotransmitter glutamate, which may arise in response to a cross-reactive microbial antigen. Treatment with either TPE or IVIG has been followed by temporary improvement.<sup>20</sup> Sydenham chorea is a movement disorder that may follow a group A streptococcal infection in children. Patients with Sydenham chorea may also exhibit obsessive-compulsive symptoms, while children who develop obsessive-compulsive behaviors, tics, and other neurologic symptoms in the absence of chorea may also have evidence of streptococcal infection (pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection; PANDAS).<sup>52</sup> A cross-reactive antibody response to streptococcal antigens could mediate symptoms in some of these patients, and small controlled trials have indicated that both TPE and IVIG can be beneficial in either Sydenham chorea or PANDAS.<sup>20,21(p143),53</sup>

### Multiple Sclerosis

Multiple sclerosis (MS) is a disease characterized by localized neurologic dysfunction that is caused by demyelinated

“plaques” in the CNS. Two clinical patterns are observed. About 70% of patients will have acute attacks that resolve fully or partially (relapsing-remitting). The other 30% have slow, continual progression of disease (chronic progressive). The frequency of attacks in relapsing-remitting MS tends to decrease with disease duration, and some patients evolve to a chronic progressive pattern.<sup>54</sup>

Discrete areas or plaques of demyelination in white matter, which are easily visualized with magnetic resonance imaging (MRI), are the hallmarks of MS. Histopathologically these areas are initially inflammatory but progress to fibrosis.<sup>54</sup> The mechanism of their appearance remains unexplained, but most authorities in the field suspect involvement of the immune system.<sup>55</sup> Experimental allergic encephalomyelitis, an animal model of MS, can be induced by immunization with myelin basic protein (MBP) or other myelin proteins. Experimental allergic encephalomyelitis is mediated by T cells and can be passively transferred with T cells from immunized animals. Most evidence suggests a misdirected cellular immune response in MS as well, and it has been difficult to assign a primary pathogenic role to circulating antibody. Antibodies to MBP or myelin oligodendrocyte glycoprotein (MOG) can be found both in CNS lesions<sup>56</sup> and in serum<sup>57</sup> in some MS patients. Although they are predictive of progression to MS in patients with a clinically isolated demyelinating event,<sup>58</sup> such antibodies do not produce MS in experimental animals.<sup>59</sup> Also, anti-MOG does not correlate with histopathologic classification or apparent responsiveness to TPE.<sup>60</sup>

The study of treatments for MS is complicated by the natural history of the disease, particularly the tendencies for acute attacks to subside and for attack frequency to decline, but also by other spontaneous fluctuations in disease activity. Clinical measurement tools such as the Expanded Disability Status Scale are used to quantify improvement or progression of disability. Although these bring some degree of objectivity to clinical studies, they are subject to interrater variability.<sup>61,62</sup>

Immunosuppressive and immunomodulatory agents have been the mainstays of drug therapy in MS. Resolution of acute attacks is thought to be hastened by brief courses of either glucocorticoids or adrenocorticotrophic hormone, both of which may promote faster restoration of normal nerve conduction by decreasing edema and inflammation in and around new plaques. The relentless progression of disability is probably not halted by these measures, although aggressive treatment of optic neuritis with intravenous steroids may delay the onset of frank MS that often follows. Cyclosporine, total lymphoid irradiation, and cytotoxic immunosuppressants such as azathioprine and cyclophosphamide have only modest benefits that may not warrant the risks such drugs entail. Mycophenolate mofetil and low dose methotrexate have also been tried. Mitoxantrone and interferon- $\beta$  have shown promise in recent studies, as has glatiramer acetate, a mixture of synthetic polypeptides that may modulate immune responses to MBP. These agents reduce the frequency of acute attacks and the appearance of new lesions seen with MRI. Immunomodulatory

monoclonal antibodies, such as alemtuzumab, daclizumab, natalizumab, and rituximab are also being investigated.<sup>62-64</sup>

Prophylactic administration of IVIG to patients with MS is reported to reduce the frequency of attacks and may slow clinical deterioration in relapsing-remitting patients. It has not as yet been shown to mediate improvement in chronic visual impairment or motor symptoms.<sup>64</sup>

The rationale for TPE in MS is uncertain, given the paucity of evidence that any circulating factor has a role in the etiology of acute attacks or chronic progression. Therapeutic plasma exchange has nevertheless been used, and encouraging results have been reported from uncontrolled studies. In controlled trials, however, it has been difficult to discern benefit, even with vigorous TPE regimens.<sup>14</sup> The first randomized, double-blind, sham-controlled study in chronic progressive MS was reported to show significant benefit for patients receiving TPE in addition to cyclophosphamide and prednisone.<sup>65</sup> The study was subsequently questioned because of anomalies in statistical analysis, and because dramatic recoveries in several of the TPE patients, not seen in subsequent trials, suggested that some relapsing-remitting patients were misclassified and entered into the trial during attacks that would have improved spontaneously.<sup>61</sup> Two later sham-controlled trials have not shown convincing benefit.<sup>66,67</sup> A single trial suggested that TPE could be useful in a subset of patients with MS with prolonged severe demyelination<sup>68</sup>; however, that study combined results of MS patients with those of patients having neuromyelitis optica. The Therapeutics and Technology Assessment subcommittee of the American Academy of Neurology and the MS Council for Clinical Practice Guidelines have classified TPE as “of little or no value” in progressive MS and of uncertain value in acute severe demyelination in previously non-disabled individuals.<sup>69</sup>

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## Therapeutic Plasma Exchange in Hematologic and Oncologic Disorders

Therapeutic plasma exchange has been tried in a variety of hematologic and oncologic conditions. These are listed in Table 42-4 along with their respective indications and intensity categories.

### Monoclonal Proteins

In addition to peripheral neuropathy (discussed above), four other syndromes associated with monoclonal immunoglobulins are regarded as clinical indications for TPE. Hyperviscosity, coagulopathy, and renal failure, which almost always occur in the setting of a malignant B-cell disorder, are discussed below; cryoglobulinemia is covered in a later section.

Hyperviscosity was the first condition to be treated successfully with manual plasmapheresis, the precursor to TPE. The full-blown syndrome consists of neurologic symptoms, a bleeding diathesis, a peculiar retinopathy marked by alternating dilated and constricted segments in retinal veins, and hypervolemia caused by expansion of plasma volume. Symptoms are uncommon if the

**Table 42-4.** Indication and Intensity of Treatment Categories for TPE in Hematologic and Oncologic Disorders

| Disorders                                       | Antibody Specificities | ASFA Indication Category* | Intensity Category† |
|-------------------------------------------------|------------------------|---------------------------|---------------------|
| Hyperviscosity syndrome                         | Not applicable         | I                         | P, C                |
| Hemolytic disease of the fetus and newborn      | Rh(D) or other         | II                        | R                   |
| ABO-incompatible marrow transplant              | A or B                 | II                        | A                   |
| Platelet alloimmunization and refractoriness    | HLA antigens           | NR                        | R                   |
| Thrombotic thrombocytopenic purpura             | vWF-cleaving enzyme    | I                         | A                   |
| Hemolytic uremic syndrome                       | Unknown                | III                       | A                   |
| Posttransfusion purpura                         | HPA-1a or other        | III                       | A                   |
| Idiopathic thrombocytopenic purpura             | Unknown                | IV (II for IA)            | R                   |
| Autoimmune hemolytic anemia                     | Unknown                | III                       | R                   |
| Aplastic anemia                                 | Unknown                | III                       | R                   |
| Pure red cell aplasia                           | Unknown                | III                       | R                   |
| Coagulation factor inhibitors                   | Factor VIII or other   | III                       | A                   |
| Catastrophic antiphospholipid antibody syndrome | Phospholipids          | III                       | A, R                |

\*I = standard first-line therapy; II = second-line therapy; III = controversial; IV = no efficacy.

†A = aggressive; R = routine; P = prolonged; C = chronic (see Table 42-2).

TPE = therapeutic plasma exchange; ASFA = American Society for Apheresis; vWF = von Willebrand factor; HPA = human platelet antigen; IA = protein A-silica immunoadsorption; NR = not rated.

relative serum viscosity is below 4 and become more likely when it exceeds 6. The hyperviscosity syndrome is most often seen in patients with Waldenström's macroglobulinemia, who have IgM paraproteins, but it may also occur in multiple myeloma.<sup>70</sup>

At higher paraprotein levels a relatively large change in viscosity may follow a relatively small change in concentration. It is this nonlinear relationship that allowed the 2-unit manual plasmapheresis technique available in the 1950s to lower viscosity enough to relieve symptoms. Because most IgM paraproteins are roughly 80% intravascular, the same relationship also predicts that a one-plasma-volume automated exchange will provide a wide margin of safety and can therefore be repeated less frequently for hyperviscosity than is necessary for many other conditions. Viscosity measurements should guide therapy, of course, but treatment every 1 to 2 weeks may be adequate.<sup>70</sup>

Paraproteins may interfere in platelet and clotting factor interactions in the absence of hyperviscosity. Such coagulopathies are found in 60% of patients with macroglobulinemia, 40% of patients with IgA myeloma, and 15% of patients with IgG myeloma.<sup>71</sup> In instances that are clinically significant, TPE therapy can help restore adequate hemostasis.

Renal failure develops in 3% to 9% of patients with myeloma and confers a poor prognosis. In many cases, renal biopsy demonstrates accumulation of free light chains in renal tubules. Urinary excretion of light chains will greatly exceed the amount that could be removed by TPE if renal function is normal. The reverse may be true in renal failure; however, light chain levels return to baseline within hours after TPE.<sup>72</sup> Three controlled studies have examined rates of recovery of renal function in myeloma patients who received TPE therapy. In one study, three of seven dialysis-dependent patients who received TPE recovered renal function, while none

of five control patients did.<sup>73</sup> In another, 13 of 15 patients in the treatment group recovered renal function compared with only two of 14 controls.<sup>74</sup> A more recent controlled study enrolled 104 patients and assessed a composite outcome comprising death, dialysis, or a low glomerular filtration rate. Outcomes were not improved in the patients randomly assigned to receive TPE treatment.<sup>75</sup> Thus, the value of TPE in this circumstance is questionable.

### Alloantibodies to Blood Cells

Alloantibodies to red cells and platelets may be problematic in a number of disease processes. Treatment by antibody removal has been tried in several of these.

Hemolytic disease of the fetus and newborn was one of the first problems to be approached with automated apheresis instruments. In the hope of slowing fetal hemolysis, sensitized D-negative mothers carrying D-positive fetuses underwent TPE to lower anti-D titers. Since the original reports, the number of sensitized mothers has declined as prophylaxis with Rh Immune Globulin has become more widespread. Furthermore, intrauterine transfusion with D-negative red cells has proven to be a better treatment for affected fetuses. For these reasons, TPE is seldom called for now, but it may still be useful in an occasional pregnancy when there is very early evidence of fetal involvement, because intrauterine transfusion is not feasible before about 18 weeks of gestation.<sup>76</sup> Prompt, early institution of TPE was followed by successful delivery after multiple prior abortions in compelling case reports involving mothers with anti-M and anti-P.<sup>18</sup>

Therapeutic plasma exchange has also been used to remove isoagglutinins in the setting of hematopoietic stem cell transplantation. Allogeneic transplantation across a major ABO barrier (eg, group A donor to group O recipient) is feasible if a

hemolytic transfusion reaction to red cells in the transplant can be avoided. This was first accomplished in marrow transplants by exhaustive pretransplant TPE of the recipient; however, most centers have preferred to simply remove most of the red cells (eg, <20 mL remaining) from an incompatible marrow graft before transplantation.<sup>77</sup> Peripheral blood stem cell collections include far fewer red cells (usually <10 mL). Such grafts can be infused safely without any manipulation. Red cell engraftment may be delayed in this situation. Therapeutic plasma exchange has been used, both before transplant to avoid delayed engraftment and after transplant to correct it, with uncertain results in both cases.<sup>78</sup> Transplantation of a solid organ, such as a liver or kidney, across a major ABO barrier can result in hyperacute rejection and is usually avoided for this reason. When organ availability is limited, however, clinical circumstances have sometimes led to transplantation of an ABO-incompatible liver or kidney after extensive TPE of the recipient, often with a satisfactory outcome.<sup>19,79</sup>

Organ transplantation across a minor ABO barrier (eg, group O donor to group A recipient) does not carry an increased risk of rejection. Some patients, however, develop hemolytic anemia caused by isoagglutinins derived from B lymphocytes in the transplant. Red cell destruction mediated by these “passenger lymphocytes” is most often seen in heart-lung or liver transplantation, in which the volume of lymphoid tissue transplanted is relatively high, and is usually evident by 1 to 3 weeks after transplantation.<sup>80</sup> Severe hemolysis may improve after TPE and compatible (eg, group O) red cell transfusion.<sup>81</sup>

Alloimmunization to HLA or platelet antigens can cause refractoriness to platelet transfusions. To restore responsiveness, antibody removal by means of TPE or a protein A-silica column has been attempted. Intravenous immune globulin has also been tried.<sup>18</sup> However, the results have been inconclusive and the best option for alloimmunized patients is transfusion of compatible platelets.

## Thrombotic Thrombocytopenic Purpura and Hemolytic Uremic Syndrome

### Thrombotic Thrombocytopenic Purpura

Thrombotic thrombocytopenic purpura is characterized by microangiopathic hemolytic anemia and thrombocytopenia, often severe. Central nervous system changes, fever, and renal abnormalities may be seen in advanced cases, although frank renal failure is unusual. A rare relapsing form begins in childhood, but the majority of cases in adults are sporadic, with women accounting for 70%. Thrombotic thrombocytopenic purpura is most often idiopathic but may be seen in association with other illnesses, such as systemic lupus erythematosus (SLE) and HIV infection, as well as with certain drugs. Idiopathic TTP was formerly assigned a mortality rate of 90%, but empiric studies in the 1970s and 1980s demonstrated much improved survival in patients receiving daily TPE with plasma replacement. Some patients, including children with the relapsing form of the disease, will respond to simple plasma infusion.<sup>82</sup>

A convincing account of the pathogenesis of TTP has been offered. It involves a plasma enzyme (a metalloproteinase called ADAMTS13 on the basis of structural features) that cleaves ultralarge von Willebrand factor (ULvWF) multimers secreted by endothelial cells, yielding the smaller vWF polymers found in normal plasma. Children with relapsing TTP have an inherited deficiency of ADAMTS13,<sup>83</sup> while in idiopathic adult cases an “acquired deficiency” arises because of the formation of an autoantibody inhibitor to the enzyme.<sup>84,85</sup> In either case, persistence of ULvWF in the circulation promotes inappropriate adherence of platelets to endothelial cells and to each other, leading to consumptive thrombocytopenia and to microvascular obstructions that cause mechanical trauma to red cells and varying degrees of end-organ ischemia. Periodic plasma infusion may abort or prevent attacks in congenitally deficient patients by supplying active enzyme.<sup>83</sup> Idiopathic cases respond better to TPE (78% response rate vs 63% for plasma infusion in the Canadian study),<sup>86</sup> presumably because exchanges remove inhibitory antibody as well as replacing the deficient enzyme. Exchanges are usually carried out daily until the platelet count and LDH (as a marker of hemolysis) have normalized.

Various immunosuppressive maneuvers, including glucocorticoid therapy, vincristine, rituximab, and splenectomy have been advocated as adjunctive treatments. Discovery of a causative autoantibody provides support for their use in idiopathic, but not in congenital, cases.<sup>18,21(p159)</sup>

A syndrome resembling TTP has been identified in some patients taking the antiplatelet drugs ticlopidine<sup>87</sup> and clopidogrel.<sup>88</sup> Antibody to ADAMTS13 has been detected in such cases,<sup>89</sup> and TPE has been reported to improve outcome (76% survival vs 50% for unexchanged patients in a retrospective study of ticlopidine recipients).<sup>87</sup> ADAMTS13 deficiency is generally not found in TTP-like syndromes associated with other drugs such as quinine or cyclosporine.<sup>90</sup>

### Hemolytic Uremic Syndrome

The hemolytic uremic syndrome (HUS) usually involves microangiopathic hemolysis, renal insufficiency, and mild-to-moderate thrombocytopenia. It occurs in children in a self-limited form that follows infection with a verotoxin-producing *Escherichia coli* (diarrhea positive); however, not all pediatric cases have this association (diarrhea negative). Both a familial and a nonfamilial form may be seen in adults. Some nonfamilial cases are associated with prior chemotherapy or stem cell transplantation.

Because of the striking overlap in clinical manifestations, it was long supposed that the pathogenesis of HUS was the same as, or similar to, that of TTP. Largely on this basis, TPE and protein A-column therapy have been recommended for diarrhea-negative childhood HUS and for adult HUS. Responses have generally been less favorable than in TTP, especially in HUS associated with chemotherapy or hematopoietic stem cell transplantation.<sup>82,91,92</sup>

Studies of ADAMTS13 in adult patients with familial and nonfamilial forms of HUS have revealed neither severe deficiency nor inhibitory activity.<sup>84</sup> Thus, the hypothesis that HUS has the same pathogenic mechanism as TTP would seem to be in error. Deficiency of complement factor H has been identified in some patients with familial HUS.<sup>93</sup> This might provide some rationale for plasma replacement therapy; however, for most cases of HUS the role of TPE is unclear at this time. This is also true for the role of TPE in the HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet count) in pregnant women,<sup>94</sup> which shares clinical features with TTP, HUS, and preeclampsia.

### Posttransfusion Purpura

Posttransfusion purpura (PTP) is a rare syndrome in which the platelet count falls to dangerously low levels about 1 week after an allogeneic transfusion. Patients who develop the syndrome lack the common allele for one of the platelet-specific glycoprotein antigens, most often the HPA-1a antigen on glycoprotein IIIa. Most patients have had multiple prior transfusions or pregnancies, and have been immunized thereby to the platelet-specific antigen coded by the prevalent allele. The transfusion that precedes the illness appears to stimulate an anamnestic increase in the titer of IgG platelet-specific alloantibody, most often anti-HPA-1a. Posttransfusion purpura is self-limited and should resolve without treatment after a few weeks; however, bleeding complications, including fatal CNS hemorrhage, may occur in the interim.<sup>95</sup>

Although it is somehow linked to a platelet-specific alloantibody response, the mechanism of extensive destruction of antigen-negative autologous platelets in PTP remains uncertain. The following four possibilities have been proposed: 1) immune complexes consisting of platelet antigen and platelet antibody bind to autologous platelets and mediate their destruction, 2) soluble platelet-specific alloantigen derived from the transfusion is adsorbed by autologous platelets, 3) a simultaneous platelet autoantibody response occurs, or 4) a broad polyclonal alloimmune response produces some antibodies that cross-react with autologous platelets.

Treatment of PTP is recommended, because many patients will have bleeding complications. High-dose glucocorticoids are usually given empirically and pulse methylprednisolone at 1 g/day has been reported effective. Platelet transfusions, even those from antigen-negative donors, seldom raise the platelet count but are likely to cause severe reactions. Daily TPE usually promotes a rise in platelet count within several days and is thought to be an effective treatment for this reason, even though no controlled trials have been conducted.<sup>96</sup> Exchanges usually include FFP replacement to avoid a superimposed humoral coagulopathy. Intravenous immune globulin produces a similarly rapid increase in platelet count and has become the favored treatment modality for this group of patients.<sup>97</sup>

### Idiopathic Thrombocytopenic Purpura

Idiopathic thrombocytopenic purpura (ITP) is an autoimmune illness affecting platelets. Most patients have an autoantibody of

the IgG class directed against a platelet-membrane glycoprotein antigen. Idiopathic thrombocytopenic purpura is sometimes accompanied by warm autoimmune hemolytic anemia (WAIHA; Evans syndrome). In pediatric patients, ITP is acute and self-limited; recovery is the rule regardless of treatment. Adults with ITP, most of whom are women, seldom recover without treatment and their disease often progresses to a chronic form. An ITP syndrome may also be seen in patients with SLE or HIV.<sup>98,99</sup>

The goal of treatment in ITP is to prevent bleeding. Fortunately most of the circulating platelets in patients with ITP are relatively young and have above-average hemostatic activity, so that a normal platelet count is not needed to avoid hemorrhage. Glucocorticoids, splenectomy, and IVIG are the mainstays of therapy.<sup>98,99</sup> Large intravenous doses of anti-D will often raise the platelet count in Rh(D) positive patients because excessive red cell destruction occupies phagocytic cells and inhibits platelet destruction. A few favorable anecdotal reports of TPE in ITP appeared in the 1970s, and one small trial suggested a lower rate of splenectomy in exchanged patients but a later study found no long-term benefit associated with such therapy.<sup>99</sup> Given this experience, enthusiasm for this approach has waned.<sup>21(p134)</sup>

Favorable responses to protein A-silica column treatment have been reported in ITP associated with HIV infection,<sup>100</sup> as well as in patients with chronic ITP without HIV.<sup>101</sup> The mechanism of action of protein A in this effect is unknown. A purely subtractive mechanism (ie, removal of platelet antibody) seems unlikely, because responses have been reported when as little as 250 mL plasma per week was treated. Bearing this uncertainty in mind, the protein A-silica column remains an option for chronic ITP refractory to more standard therapies,<sup>21(p134)</sup> and is discussed in greater detail in Chapter 41.

### Autoimmune Hemolytic Anemia

Autoimmune hemolytic anemia (AIHA) is caused by autoantibodies to red cells. Such antibodies are classified as either “cold” or “warm” agglutinins, depending on the temperature of maximal activity. Cold agglutinins are usually IgM antibodies directed against the I/i antigens; they bind most strongly at low temperatures and may produce a syndrome of complement-mediated intravascular hemolysis (cold agglutinin disease; CAD). Warm agglutinins are usually IgG and are often directed against an antigen that does not appear on Rh<sub>null</sub> cells; they bind better at body temperature and produce a predominantly extravascular hemolytic syndrome, WAIHA. Autoimmune hemolytic anemia can be idiopathic but can also be associated with infections, lymphoproliferative disorders, or other autoimmune diseases.<sup>102</sup>

Most patients need treatment. Standard therapy is aimed at lowering antibody production and inhibiting destruction of sensitized cells. Glucocorticoids, IVIG, and splenectomy are often effective in WAIHA, and other immunosuppressive drugs may be tried if these measures fail. All of these approaches are less successful in CAD.<sup>102</sup>

Therapeutic plasma exchange to deplete circulating antibody has been tried in both WAIHA and CAD when conventional

treatments have failed. Because the IgM antibodies in CAD are predominantly intravascular and only loosely bound to cells, their removal by TPE should be relatively efficient. Such therapy, when added to conventional drug treatment, has been reported to lower antibody titers and transfusion requirements, albeit only temporarily. In WAIHA much of the circulating antibody is bound to red cells; TPE has been tried in this disorder also, but it is less likely to be helpful.<sup>21(p118)</sup> A nonrandomized study compared responses to red cell transfusions in five AIHA patients who underwent TPE before some transfusions and four other patients who did not. Responses were significantly better in the patients who did *not* receive TPE and were not improved by TPE in the patients who did, suggesting that TPE is not effective for this purpose.<sup>103</sup>

### Pure Red Cell Aplasia and Aplastic Anemia

Pure Red Cell Aplasia and Aplastic Anemia are marrow disorders. In the former there is reticulocytopenic anemia, while the latter leads to pancytopenia. At least some cases of both conditions likely have an immunologic basis. Allogeneic marrow transplantation is the preferred treatment for severe aplastic anemia if a suitable donor is available, but immunosuppressive therapies, such as glucocorticoids, cytotoxic drugs, cyclosporine, and antithymocyte globulin may be effective, especially in milder cases.

In the serum of a minority of patients it is possible to demonstrate a factor, probably antibody, that inhibits the growth of marrow-derived precursor cells in culture.<sup>104</sup> This provides a rationale for TPE, which has been reported in both disorders in a few cases. Results in aplastic anemia have been mixed; responses appear to be more likely in patients with serum inhibitory activity. All reported instances of TPE treatment for pure red cell aplasia have led to improvement, which is sometimes quite dramatic in patients with serum inhibitory activity. Thus, although TPE is not a primary therapy for either disorder, it can be offered to patients who have failed to improve after receiving conventional treatment, especially those found to have serum inhibitory factors.<sup>21(p117)</sup>

### Coagulation Factor Inhibitors

Coagulation factor inhibitors are IgG antibodies to components of the clotting cascade. They interfere with clotting by inactivating the targeted factor. Inhibitors may be autoantibodies that arise in individuals with no prior bleeding. Alternatively they may be alloantibodies that form in genetically deficient patients after exposure to “foreign protein” in the course of factor replacement therapy. Factor VIII is the clotting protein most often affected by antibodies of either type, and most of the following concerns Factor VIII inhibitors.

The two goals of treatment for a patient with an inhibitor are control of individual bleeding episodes and suppression of inhibitor synthesis. Depending on inhibitor titer, the first goal can sometimes be achieved by infusion of high doses of human Factor VIII. Porcine Factor VIII, which cross-reacts

only partially with human Factor VIII antibodies, can be effective in the face of somewhat higher inhibitor titers, but its availability in recent years has been uncertain. For patients with the highest titers, Factor VIII-bypassing products such as recombinant Factor VIIa are needed. Therapeutic plasma exchange or selective immunoadsorption of IgG during a bleeding episode may reduce inhibitor titers enough to allow infused Factor VIII to bring about hemostasis. Suppression of inhibitor synthesis is approached with immunosuppressive measures, including high-dose glucocorticoids, cytotoxic agents, cyclosporine, IVIG, and rituximab.<sup>15,105,106</sup> Tolerance-inducing protocols that include regular infusion of exogenous Factor VIII have been devised for patients with alloimmune inhibitors. In the so-called Malmö protocol, extensive TPE or IgG depletion with protein A-sepharose column procedures is used to reduce the inhibitor level at the onset of treatment so that infused “tolerizing” factor can circulate.<sup>107</sup> Frequent, large (two to three plasma volume) exchanges with FFP replacement are recommended for patients with inhibitor. Central venous access is often required; placement of a catheter in a patient with a refractory bleeding diathesis is a challenge for all concerned and often mandates infusion of a Factor VIII-bypassing product for wound hemostasis.<sup>108</sup>

Therapeutic plasma exchange has also been reported for treatment of patients with antiphospholipid antibodies, which may interfere with in-vitro assays of coagulation, such as the partial thromboplastin time. In contrast to the inhibitory antibodies described above, however, they usually promote inappropriate coagulation in vivo and cause thrombotic events. Patients suffering thrombotic events in three or more organ systems are said to have catastrophic antiphospholipid antibody syndrome.<sup>109</sup>

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## Therapeutic Plasma Exchange in Rheumatic and Other Immunologic Disorders

Therapeutic plasma exchange has been tried in a number of rheumatic diseases and other diseases that are considered to have an immune or autoimmune etiology. These are listed in Table 42-5 along with the author's intensity guidelines and the indication categories assigned by ASFA.

### Cryoglobulinemia

Cryoglobulins are abnormal serum proteins that precipitate reversibly at 4°C; some will precipitate at higher temperatures. Such precipitates always contain immunoglobulin, and immunoelectrophoretic or immunofixation analysis allows distinction of three types. Type I cryoglobulins consist of a single species of monoclonal immunoglobulin. These are usually found in B-cell lymphoproliferative disorders such as myeloma or Waldenström's macroglobulinemia. Cryoglobulin levels are often quite high (>500 mg/dL) and may cause Raynaud phenomenon or acral necrosis due to microvascular obstruction, as well as other symptoms. Type II cryoglobulins contain both monoclonal and polyclonal immunoglobulins. The former is usually an IgMκ

**Table 42-5.** Indication and Intensity of Treatment Categories for TPE in Rheumatic and Other Immunologic Disorders

| Disorders                              | Antibody Specificities | ASFA Indication Category* | Intensity Category† |
|----------------------------------------|------------------------|---------------------------|---------------------|
| Cryoglobulinemia                       | IgG                    | II                        | A, R, P, C          |
| Rheumatoid arthritis                   | IgG                    | NR (II for IA)            | R ?                 |
| Systemic lupus erythematosus           | dsDNA and others       | III (IV for nephritis)    | R, P                |
| Systemic vasculitis                    | ANCA                   | NR                        | R ?                 |
| Polymyositis and dermatomyositis       | Unknown                | IV                        | R?                  |
| Goodpasture syndrome                   | Type IV collagen       | I                         | A                   |
| Rapidly progressive glomerulonephritis | ANCA                   | II                        | R                   |
| Renal transplantation                  |                        |                           |                     |
| Humoral rejection                      | HLA antigen            | II                        | A, R                |
| Presensitization                       | HLA antigen            | II                        | R                   |
| Recurrent focal glomerulosclerosis     | Unknown                | III                       | R                   |
| Heart transplant rejection             | HLA antigen            | III                       | R                   |

\*I = standard first-line therapy; II = second-line therapy; III = controversial; IV = no efficacy; IA = protein A-silica immunoadsorption, NR = not ranked.

†A = aggressive; R = routine; P = prolonged; C = chronic (see Table 42-2).

TPE = therapeutic plasma exchange; ASFA = American Society for Apheresis; IgG = immunoglobulin G; dsDNA = double-stranded DNA; ANCA = antineutrophil cytoplasmic antibodies; NR = not rated.

with anti-IgG specificity, while the latter is polyclonal IgG bound to the IgMκ in an immune complex. Most cases occur in the context of chronic hepatitis C infection. They typically manifest a cutaneous vasculitis on the lower extremities and may have visceral manifestations of immune complex disease as well.<sup>110</sup> Type III cryoglobulins are mixed polyclonal, often with IgM anti-IgG that binds IgG in immune complexes. These may arise in acute infections, such as hepatitis B, or in chronic inflammatory states such as severe rheumatoid arthritis. Clinical manifestations resemble serum sickness.

If there is an underlying condition, cryoglobulin levels and related symptoms may decrease with treatment of this primary disorder, for example, chemotherapy for myeloma or interferon for hepatitis C virus infection. For idiopathic and secondary cases of mixed cryoglobulinemia, prednisone therapy often relieves symptoms, while alkylating agents may be useful in patients with severe symptoms resistant to prednisone.

Therapeutic plasma exchange will reduce cryoglobulin levels and control symptoms, even in the absence of other treatments,<sup>111,112</sup> but inconvenience and expense mitigate against such use. It should be started promptly for patients who seek treatment for severe acral ischemia or visceral manifestations of vasculitis, in whom it can help achieve control of symptoms until aggressive drug therapy takes hold.<sup>113</sup> Patients with chronic vasculitic skin ulcers may also benefit.<sup>114</sup> In all cases replacement fluids should be warmed to body temperature before infusion.

### Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a disease of unknown cause that is more prevalent in women. It is the most common chronic inflammatory joint disease and a leading cause of disability. Most patients have rheumatoid factor, an IgM autoantibody to IgG; however, because this antibody is absent in many clinically typical

cases, and because it is also found in patients who do not have arthritis, it is not likely to be directly involved in pathogenesis.

Conservative treatment includes nonsteroidal antiinflammatory agents, oral glucocorticoids in low doses, and intraarticular steroids. More severely affected patients eventually receive slow-acting “disease-modifying antirheumatic drugs” that are probably immunomodulatory, such as antimalarials, gold compounds, and methotrexate. Tumor necrosis factor and IL-1 inhibitors are also approved for treatment of RA.

Therapeutic plasma exchange was tried for RA in the 1970s and 1980s, but controlled trials did not show benefit. There were subsequent reports of lymphapheresis, with or without accompanying TPE. Some controlled trials investigating this approach showed significant but short-lived benefit, while others did not. In practice, the prospect of only a modest chance of modest benefit from a costly, inconvenient therapy has discouraged treatment with therapeutic apheresis.<sup>17</sup>

A sham-controlled trial of 12 weekly protein A-silica column treatments produced improvement in 33% of 48 treated patients vs only 9% of 43 controls. Benefit persisted for about 8 months on average, and a subsequent course was again beneficial in seven of nine initial responders.<sup>115</sup> Although its mechanism of action is unclear, this device has gained Food and Drug Administration approval for use in RA.

### Systemic Lupus Erythematosus

Systemic lupus erythematosus has long been regarded as the prototypic autoimmune disease. The most important diagnostic criterion, circulating antibodies to DNA, especially double-stranded DNA (anti-dsDNA), identifies patients who may have a variety of other autoantibodies and a disparate array of clinical syndromes in which skin disease, joint disease, cytopenias, or nephritis may be the sole or dominant problem.

Immunosuppressive measures are the cornerstone of therapy for SLE. Most patients are given prednisone in varying doses, and those with severe disease may also receive azathioprine or cyclophosphamide. The plethora of autoantibodies that seem relevant to clinical signs made SLE an obvious target for TPE. It was one of the first illnesses to be treated with automated TPE in the early 1970s, and early case reports and uncontrolled series suggested a favorable effect.<sup>17</sup>

Lupus nephritis is a particularly devastating manifestation in which glomerular deposition of immune complexes and anti-dsDNA is believed to have a prominent role in pathogenesis. Thus it seemed an attractive setting for randomized trials of TPE. A controlled trial with only eight patients suggested benefit.<sup>116</sup> However, in a multicenter randomized controlled trial comparing oral cyclophosphamide plus TPE to oral cyclophosphamide alone, there was no advantage for the patients receiving TPE.<sup>117</sup> A later international trial, which enrolled patients with a variety of severe manifestations, was structured to exploit enhanced sensitivity to a properly timed pulse dose of intravenous cyclophosphamide that was believed to follow pathogenic antibody removal by TPE.<sup>118</sup> As mentioned above, subsequent work with knock-out mice deficient for the FcRn receptor suggests that enhanced susceptibility would not occur.<sup>4</sup> In any case, this trial also failed to show any advantage for all patients treated with TPE<sup>119</sup> or for a subgroup with nephritis.<sup>120</sup> Thus large controlled studies have failed to confirm any worthwhile effect of TPE in SLE.

### Systemic Vasculitis

The term *systemic vasculitis* encompasses a group of disorders that cause inflammation in blood vessel walls and ischemic tissue damage. Vasculitis syndromes are conveniently classified on the basis of the size of vessels typically involved, but most are of unknown etiology. Immune complexes are found in patients with some syndromes, and autoantibodies such as antineutrophil cytoplasmic antibodies (ANCA) in Wegener's granulomatosis (c-ANCA) and polyarteritis (p-ANCA), can be demonstrated in others. This has lent credence to the notion that humoral immune factors are somehow involved.<sup>121</sup>

Prednisone is the first-line therapy for most vasculitic syndromes and cyclophosphamide is often added in more severe cases. Randomized controlled trials in renal vasculitis,<sup>122</sup> as well as in a group of patients with polyarteritis or Churg-Strauss angiitis,<sup>123</sup> have shown little evidence that addition of TPE to drug therapy confers long-term benefit. Nevertheless, it may be requested for patients who are not responding to maximal drug therapy.

### Polymyositis and Dermatomyositis

Polymyositis and dermatomyositis are inflammatory diseases affecting skeletal muscle. A characteristic dermatitis involving the eyelids, knuckles, neck, and shoulders is also part of the latter condition. The usual clinical picture includes proximal weakness with biochemical evidence of muscle cell enzyme leakage; the diagnosis is confirmed by muscle biopsy. The natural

history is progressive fiber loss, eventually leading to profound, irreversible weakness. An autoimmune etiology is suspected, but circulating antibody specific for skeletal muscle has not been implicated. Initial treatment is high-dose prednisone, which can often be tapered to maintenance levels. Resistant disease is treated with azathioprine, methotrexate, an alkylating agent, or a combination. Controlled trials have also shown that IVIG infusion reduces muscle enzyme levels and improves strength temporarily.<sup>20</sup>

Several uncontrolled series were interpreted to show that TPE was beneficial, but they were unfortunately confounded by concurrent escalations in immunosuppressive drug therapy.<sup>20</sup> A randomized controlled trial in which 12 patients received TPE, 12 received lymphapheresis, and 12 received sham apheresis, with no changes in drug therapy, showed no difference in the response rate among the three groups.<sup>124</sup> Thus despite the successes with IVIG, there appears to be no role for TPE in the treatment of polymyositis.

### Goodpasture Syndrome

Goodpasture syndrome (GPS) is characterized clinically by pulmonary hemorrhage and rapidly progressive glomerulonephritis. Light microscopy of renal biopsies shows crescent formation in many glomeruli, while immunofluorescent and electron microscopy reveal linear subendothelial immune deposits that may also be evident in a lung biopsy. In 95% of cases there is a circulating antibody that binds to glomerular basement membrane (anti-GBM). Such antibodies are specific for a noncollagenous sequence near the carboxy terminus of the  $\alpha 3$  chain of type IV collagen, which is found in appreciable quantities only in renal and pulmonary basement membranes. Untreated GPS progresses quickly and relentlessly, and most patients die of uremia or complications of lung hemorrhage.<sup>125</sup> The preferred treatment for GPS is high-dose prednisone and cyclophosphamide, combined with aggressive TPE, to quickly reduce anti-GBM levels and minimize progression of tissue damage.<sup>19</sup> Exchanges are usually carried out daily and may be continued for up to 2 weeks. It is prudent to give some FFP replacement in the latter part of each exchange to avoid a dilutional coagulopathy that might cause exacerbation of lung bleeding.

A single controlled trial failed to show an advantage for GPS patients who received TPE; however, this study has been largely discounted because the TPE schedule (every 3 days) was not sufficiently aggressive and because the extent of renal damage at entry was worse in the TPE group than in controls.<sup>126</sup> Early treatment is recommended because patients who are already dialysis-dependent at the onset of TPE are unlikely to recover renal function.<sup>127</sup> It follows that the subset of patients whose renal biopsies show irreversible lesions are not likely to benefit from TPE unless they also have pulmonary hemorrhage.

### Other Rapidly Progressive Glomerulonephritis

In addition to GPS, there are two other categories of rapidly progressive glomerulonephritis (RPGN)—those with an immune



complex disease, who have granular subendothelial immune deposits, and those with pauci-immune RPGN, who have scant immune deposits, if any. Light microscopic findings in both are similar to those found in GPS, with severe glomerular inflammation and crescent formation. Some cases also have associated lung hemorrhage. Patients in either group may have isolated renal disease or may have accompanying features that suggest a diagnosis of systemic vasculitis, mixed cryoglobulinemia, or Henoch-Schönlein purpura for granular-immune complex RPGN, or microscopic polyangiitis or Wegener's granulomatosis for pauci-immune RPGN patients, most of whom test positive for ANCA.<sup>128</sup>

Therapies are similar for these two categories of RPGN, and some trials and series have included patients with both types. Virtually all patients receive prednisone and most receive either oral or intravenous cyclophosphamide. Therapeutic plasma exchange has been used extensively in patients with both types of disease. Two controlled trials, one published in 1988<sup>129</sup> and the other in 1992,<sup>130</sup> showed no advantage for patients who received TPE in addition to immunosuppressive drugs. However, a subgroup analysis in the second study suggested that patients who have dialysis-dependent renal failure are more likely to recover renal function if they receive TPE. No such trend was noticed in the more recent trial conducted by Guillevin and colleagues.<sup>123</sup> A prospective, randomized trial by Stegmayr et al<sup>131</sup> compared TPE with immunoadsorption. Among 38 patients with non-GPS RPGN, 87% of whom had ANCA, 70% avoided long-term dialysis. Therapeutic plasma exchange and immunoadsorption were equally effective. In another study of ANCA-positive patients with an elevated creatinine, 54% of dialysis dependent patients randomly assigned to receive TPE became dialysis independent compared to only 32% of those who received pulse methylprednisolone instead of TPE.<sup>132</sup> Thus, while the role of TPE is not clearly defined in all types of RPGN, current evidence suggests efficacy in ANCA-positive patients who are dialysis dependent.

### Solid Organ Transplantation

In organ transplant recipients TPE has been used both to treat and to prevent rejection, as well as for recurrence of certain diseases in a transplanted organ. Photopheresis, which has also been tried for the same purposes, primarily in heart transplantation, is covered in Chapter 43.

### Rejection

Cellular immune mechanisms mediate most organ allograft rejection episodes; however, antibody-mediated rejection may occur rapidly in patients who have preexisting antibodies to ABO or HLA antigens expressed by the graft. Such "hyperacute" rejection is characterized histologically by neutrophil infiltration, fibrin deposition, and endothelial damage in small blood vessels; failure of the graft is mainly caused by ischemic damage. All treatments have been futile in hyperacute rejection, including TPE. So-called "vascular changes," which may be seen microscopically in later rejection episodes, were formerly taken to indicate

antibody-mediated rejection, even when immunofluorescence microscopy and tests for circulating antibody were negative.<sup>133</sup>

Standard posttransplant management for kidney and heart transplantation consists of prophylactic immunosuppression with glucocorticoids, a calcineurin inhibitor (cyclosporine or tacrolimus) and an antimetabolite such as azathioprine or mycophenolate mofetil. High-risk recipients and those who have rejection episodes in spite of these standard measures are treated with pulse steroids or T-cell antibody preparations or both.<sup>134,135</sup>

Case reports and uncontrolled series published in the late 1970s and early 1980s suggested TPE was beneficial in renal transplant rejection. Then five controlled trials<sup>19</sup> were reported in the mid- and late 1980s. Four showed no significant benefit for patients receiving TPE in addition to standard drug therapy, even in the subgroups whose transplant biopsies showed "vascular" histologic changes. In the one study suggesting benefit, the mean treatment time was 10 to 11 months after transplant, when antibody-mediated rejection is less likely. The last and largest study concluded that TPE therapy for renal transplant rejection could no longer be recommended.<sup>136</sup> Nevertheless, use of TPE for this purpose continued to be reported.<sup>19</sup>

In the more recent past, a new wave of enthusiasm for TPE has focused on patients with clinical and histologic evidence of acute rejection who also have circulating donor-specific antibody and/or deposition of complement component C4d in transplant biopsy tissue, suggesting a humoral mechanism. The antibody can be shown by a positive crossmatch with donor cells or by flow cytometric reactions with donor antigens. C4d deposition is demonstrated by immunofluorescence microscopy.<sup>133,137</sup>

Uncontrolled studies have been encouraging. Observations in the pretransplant period have shown that antibody titers can be reduced by TPE.<sup>138</sup> Furthermore, in open trials, patients with refractory acute rejection who demonstrate donor-specific antibody and/or C4d have relatively good long-term graft survival ( $\geq 80\%$ ) after treatment regimens that include TPE and IVIG.<sup>139,140</sup> A recent conference on antibody-mediated rejection recommended that demonstration of circulating antibody be considered an essential criterion for diagnosis of humoral rejection and that the effectiveness of TPE and IVIG in this entity be studied in "rigorous prospective, multicenter" trials.<sup>141</sup>

TPE has also been employed in cases of cardiac allograft rejection. Favorable outcomes have been reported in individual patients who were also receiving other therapies, but no controlled trials have been conducted. The criteria for a diagnosis of humoral rejection in this setting continue to evolve.<sup>137</sup> "Vascular" histology is more difficult to detect or exclude in the endomyocardial biopsies performed to monitor cardiac allografts because few blood vessels are found in this part of the heart muscle. Immunofluorescence microscopy for IgG had been considered a reasonable criterion for humoral rejection; however, it has been suggested more recently that this diagnosis be made, and TPE employed, in patients with deteriorating cardiac function whose biopsies lack cellular infiltrates.<sup>142</sup> In some such biopsies it has been possible to demonstrate capillary endothelial swelling, macrophage influx, and C4d

deposition.<sup>143</sup> Controlled data to support the TPE recommendation are lacking, as are any published data correlating this clinical syndrome with circulating donor-specific antibody.

Pretransplant TPE has been tried in organ transplant candidates who are presensitized to HLA antigens. Those whose sera react with lymphocytes from a large fraction of the population are less likely to have a compatible crossmatch with a cadaveric donor and hence have a lower likelihood of receiving a transplant. Prospective immunosuppression, combined with antibody removal by TPE or protein A-sepharose immunoadsorption, has been explored as a means to achieve a compatible crossmatch before transplantation and thereby prevent hyperacute rejection. Several centers have reported groups of patients who received kidney transplants after being prepared in this way and achieved quite respectable graft survival rates. Similar protocols have facilitated successful transplantation of ABO-incompatible kidneys and livers.<sup>19</sup> In more recent studies, pretransplant TPE has facilitated elective living donor transplantation of HLA-crossmatch positive as well as ABO-incompatible organs.<sup>79,138,144</sup>

In summary, evidence from controlled trials has not shown global efficacy for TPE in reversing established rejection episodes in renal allografts. Anecdotal experience focusing on patients with circulating antibody and immunopathologic evidence of humoral rejection seems more promising, but controlled trials in such patients have yet to be published. Pretransplant antibody removal can make transplantation feasible for otherwise ineligible candidates. It can also facilitate transplantation of kidneys from living donors across ABO and donor-specific crossmatch barriers. Clarification of the role of TPE in cardiac transplantation awaits controlled studies targeting patients with documented humoral rejection and circulating donor-specific antibody.

### Recurrence of Disease

Focal glomerulosclerosis (FGS) is a disease that causes nephrosis and renal failure, predominantly in children. It recurs in about 30% of allograft recipients, which suggests that a humoral factor may have a role in its pathogenesis. A 50-kD plasma factor that binds to protein A has been implicated but has not been further characterized.<sup>145</sup> Reduced proteinuria and improved renal function have been reported when recurrence in an allograft is treated with stepped-up immunosuppression and TPE.<sup>146,147</sup>

Goodpasture syndrome may occasionally recur in a transplanted kidney; however, this can usually be avoided by delaying transplantation until the anti-GBM response has subsided spontaneously. If the syndrome recurs in spite of this precaution, it should be treated promptly with TPE and cyclophosphamide.<sup>147</sup>

Allograft vasculopathy, a diffuse coronary artery disease that sometimes develops in transplanted hearts, is the leading cause of morbidity and mortality in heart transplant recipients who survive beyond 1 year. It may be related either to continuing hyperlipidemia or to chronic rejection. Selective depletion of LDLs, which is discussed in greater detail in the section on hypercholesterolemia, has been reported helpful in a few such patients with persistent lipoprotein abnormalities.<sup>148</sup>

## Therapeutic Plasma Exchange in Toxic and Metabolic Disorders

This section covers conditions in which removal of plasma constituents other than immunoglobulin is potentially beneficial. These are listed in Table 42-6 along with indication and intensity categories.

### Hypercholesterolemia

Familial hypercholesterolemia (FH) is a genetically determined deficiency of cell surface LDL receptors that interferes with cholesterol off-loading from LDL into cells and with the normal negative-feedback regulation of LDL synthesis, leading to highly elevated levels of circulating LDL, cholesterol (650 to 1000 mg/dL), and lipoprotein(a) [Lp(a)]. Skin xanthomas and coronary atheromas develop in the first decade of life in homozygotes, and death from myocardial infarction before age 20 is common. Heterozygotes also have elevated LDL, cholesterol (350 to 500 mg/dL), and Lp(a) levels; they may develop xanthomas by age 20 and coronary atherosclerosis by age 30.<sup>149,150</sup>

Milder forms of hypercholesterolemia can be influenced by dietary modifications and are amenable to drug treatment with agents such as HMG-CoA reductase inhibitors, bile acid-binding resins, nicotinic acid, and ezetimibe, a new agent that inhibits gut absorption of both dietary and biliary cholesterol.<sup>150,151</sup> However, FH homozygotes and some FH heterozygotes respond only modestly to these measures and remain at risk for premature death. Drastic surgical measures, such as ileal bypass, portacaval shunt, and liver transplantation may be recommended for such patients if they have evidence of coronary artery disease.<sup>149,150</sup> Alternatively, removal of LDL and its associated cholesterol from the blood can be accomplished repeatedly by various modalities of therapeutic apheresis.<sup>150</sup>

A standard TPE will lower LDL and cholesterol levels by 50% or more, and long-term treatment every 1 to 2 weeks can lead to shrinkage of cutaneous xanthomas and regression of coronary artery deposits. Although TPE removes both LDL and Lp(a), it also depletes high-density lipoproteins (HDL), which

**Table 42-6.** Indication and Intensity of Treatment Categories for TPE in Metabolic Disorders

| Disorders                                | ASFA Indication Category* | Intensity Category† |
|------------------------------------------|---------------------------|---------------------|
| Homozygous familial hypercholesterolemia | II (I for LDL-P)          | C                   |
| Refsum's disease                         | II                        | A, R, P             |
| Overdose or poisoning                    | III                       | A                   |
| Acute hepatic failure                    | III                       | A                   |

\*I = standard first-line therapy; II = second-line therapy; III = controversial; IV = no efficacy; LDL-P = LDL-apheresis, ie, selective depletion of LDL.

†A = aggressive; R = routine; P = prolonged; C = chronic (see Table 42-2).

TPE = therapeutic plasma exchange; ASFA = American Society for Apheresis.

are believed to have an antiatherogenic action. This disadvantage has stimulated efforts to deplete LDL semiselectively and on-line from patient plasma separated by an apheresis device, and then return the LDL-depleted plasma to the patient.<sup>150</sup> Several systems designed to accomplish this goal are described in Chapter 43.

### Refsum's Disease

Refsum's disease results from deficiency of the peroxisomal enzyme phytanoyl-CoA hydroxylase, which participates in degradation of phytanic acid by  $\alpha$ -oxidation. Accumulation of diet-derived phytanic acid in plasma lipoproteins and in tissue lipid stores leads to symptoms, which may include peripheral neuropathy, cerebellar ataxia, retinitis pigmentosa, anosmia, deafness, ichthyosis, renal failure, and arrhythmias. Slow progression is the usual course, but rapid deterioration and even sudden death may follow a marked increase in plasma phytanic acid.<sup>152</sup>

Restriction of dietary intake of phytanic acid via dairy products, meats, and ruminant fats is the mainstay of treatment. It leads to gradual clearing of phytanate stores by slow  $\omega$ -oxidation and gradual symptomatic improvement in most patients. Nutrition must be maintained, however, because mobilization of calories from endogenous fat can increase plasma phytanic acid levels acutely and cause clinical exacerbations. Therapeutic plasma exchange will remove large quantities of phytanic acid incorporated into plasma lipids.<sup>153</sup> Selective lipoprotein depletion is also effective.<sup>154</sup> Apheresis therapy is most appropriate for patients who have very high plasma phytanate levels and an associated exacerbation of symptoms. Skin disease, neuropathic symptoms, and ataxia usually improve as plasma levels drop. Cranial nerve defects usually do not.<sup>152</sup>

### Drug Overdose and Poisoning

Toxic effects may occur after exposure to excessive doses of pharmacologic agents or to harmful agents in the environment. Management techniques for both types of event are similar and may include removal of toxin still in the gastrointestinal tract, enhancement of renal elimination, and direct removal from blood by hemodialysis, hemoperfusion (eg, over charcoal columns), or TPE.<sup>155</sup> Specific antidotes may also be given if available. Serious events are usually treated with multiple measures.

Therapeutic plasma exchange has been reported to be beneficial, when combined with other therapies, in cases involving substances, such as methyl parathion, vincristine, and cisplatin, that bind tightly to plasma proteins. It has also been reported for severe hyperthyroidism, either endogenous or exogenous, in which its effectiveness may be limited by extensive binding of L-thyroxine to tissue proteins. Therapeutic plasma exchange has been reported in poisonings due to ingestion of the *Amanita phalloides* mushroom; however, diuresis clears more *Amanita* toxin.<sup>19</sup>

Unfortunately, the literature on this topic is older and entirely anecdotal. Furthermore, TPE has always been used in combination with other therapies that are presumably effective. This complicates formulation of firm, rational guidelines. Nevertheless it

seems reasonable to offer TPE to a severely affected patient with an overdose or poisoning who has a high blood level of a toxic agent that binds to plasma proteins. It should also be noted that TPE has shown minimal or no beneficial effect in overdosage of drugs known to bind to tissue proteins and lipids, including barbiturates, chlordecone, aluminum, tricyclic antidepressants, benzodiazepines, quinine, phenytoin, digoxin, digitoxin, prednisone, prednisolone, tobramycin, and propranolol.<sup>19</sup>

### Acute Hepatic Failure

Acute hepatic failure may develop after a severe liver insult, such as overwhelming hepatitis B infection or acetaminophen overdosage. Cases may also be caused by drug reactions, Wilson's disease, vascular anomalies, acute fatty liver of pregnancy, and a variety of toxins. Acute hepatic failure results in many metabolic imbalances and synthetic defects. Clinical symptoms include jaundice, coagulopathy, encephalopathy, and renal failure. The treatment of choice is liver transplantation, which leads to 60% to 80% long-term survival vs >60% mortality for untransplanted patients. Cerebral edema accounts for fatal outcomes.<sup>156</sup>

Conservative treatment is basically supportive. Fluid, electrolyte, and nutritional supplements are adjusted in response to metabolic abnormalities. Bowel sterilization with enteral antibiotics minimizes production of ammonia by intestinal bacteria. Pressors are infused if needed for hemodynamic support. Osmotic diuretics, sedatives, hyperventilation, and proper positioning are all employed to reduce intracranial pressure. Platelets and plasma products are infused to improve coagulation.<sup>156</sup>

Therapeutic plasma exchange with plasma replacement has seemed appealing as a means to restore metabolic homeostasis, remove toxic metabolites that may cause cerebral edema, and supply coagulation factors and other deficient plasma proteins in quantity without causing volume overload. Practical evaluations of this approach have produced mixed results.<sup>156</sup> Some investigators have found TPE helpful in stabilizing and maintaining patients until an organ for transplant becomes available. Improvements in blood pressure, cerebral blood flow, and neurologic status were attributed to TPE in one study<sup>157</sup>; however, intracranial pressure, a key prognostic indicator, did not decrease. Hemoperfusion over activated charcoal, which will lower plasma ammonia levels, has also shown no advantage over intensive supportive care alone.

Potential problems with extensive TPE arise from the diminished ability of patients with acute hepatic failure to metabolize the citrate in infused plasma. Accumulation of citrate leads to ionized hypocalcemia and to alterations in arterial ketone body ratios that may interfere with regeneration of hepatocytes.<sup>158</sup> Thus, although TPE can partially reverse coagulopathy and other synthetic deficits in these patients, a favorable net impact on outcome has been difficult to demonstrate.

Other methods for extracorporeal support of patients with liver failure have been investigated. Several devices place patient plasma in "metabolic contact" with hepatocyte suspensions of either human or porcine origin. One contains a suspension of

pig liver cells in the exterior space surrounding the hollow fibers in a filter cartridge. Patient plasma separated by an apheresis device and passed through a charcoal column to remove ammonia, flows through the hollow fibers before being returned to the patient. It is proposed that such devices could provide both detoxification and synthetic functions; however, evidence for the latter has been disappointing. Other devices aim to remove toxins via binding to various extracorporeal sorbents, such as activated charcoal or albumin molecules. None of these approaches has yet been shown to improve outcomes for acute liver failure patients in a large controlled trial, but research is ongoing.<sup>159</sup>

## Conclusion

Therapeutic plasma exchange is an effective therapy for a number of diseases, especially those mediated by paraproteins or autoreactive antibodies. Improvements in apheresis instruments that are outside the scope of this chapter have made it a very safe treatment as well. Thus, it should continue to have an important role in the management of selected diseases.

## Disclaimer

The author has disclosed no conflicts of interest.

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# Specialized Therapeutic Hemapheresis and Phlebotomy

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A comprehensive, evidence-based analysis of apheresis therapies by the American Society for Apheresis (ASFA) recorded 40 disease states as Category I (apheresis as first-line therapy) or Category II (apheresis as adjunctive therapy) indications for therapeutic apheresis.<sup>1</sup> Among these, 24 (60%) were indications for plasma exchange, consistent with prior surveys that found plasma exchange to be the predominant form of apheresis therapy in North America.<sup>2,3</sup> At the same time, ASFA created a new category P (“pending”) to take into account potential treatment indications that are under investigation, in particular those that are in pivotal Phase III trials that may lead to approval by the US Food and Drug Administration (FDA).<sup>1,4</sup> All of the potential indications in this category are specialized forms of apheresis that target specific fractions of the plasma or cellular compartments of the blood for processing or removal.<sup>1</sup>

Whereas specialized apheresis procedures have been in wide use for decades,<sup>5</sup> some as a result of clinical trials and some on the basis of accumulated experience,<sup>6</sup> the approval of new therapeutic indications, devices, and drugs is increasingly dependent on the presentation of carefully acquired supportive evidence.<sup>7-9</sup> This applies to apheresis therapies as well.<sup>1,10,11</sup> Accordingly, this chapter (which reviews therapeutic apheresis procedures that target, for processing, specific fractions of the plasma or cellular compartment of the blood) emphasizes those procedures that are supported by evidence.

## Therapeutic Red Cell Apheresis

John J. Abel of the Johns Hopkins University used the term “plasmapheresis” to describe a method for removing large quantities of plasma from experimental animals.<sup>12</sup> The first reported series of cases in which the manual removal of plasma was employed for clinical purposes (treatment of Waldenström’s macroglobulinemia) was described as plasmapheresis.<sup>13</sup> Thus, the

removal of red cells for therapeutic purposes can be referred to as erythrocytapheresis. This term particularly applies to the removal of red cells using automated blood processing instruments that are capable of selectively removing erythrocytes while returning the plasma, buffy coat cells, and additional isotonic saline to the patient.<sup>14-17</sup> The use of automated blood processing equipment to exchange patient red cells for donor red cells is referred to as red cell exchange,<sup>18-21</sup> although the term erythrocytapheresis is frequently used in reference to red cell exchange.<sup>1,22</sup>

## Red Cell Exchange

Therapeutic red cell exchange can be performed by manual exchange transfusion<sup>21,23(pp153-160)</sup> or with programmable automated blood processing (apheresis) instruments.<sup>18-20,22</sup> This discussion focuses on automated red cell exchange.

Basic features of automated blood processors that perform apheresis using centrifugation technology are detailed in Chapter 41. The machine operator enters the patient’s gender, height, and weight into the instrument’s computer and total blood volume is calculated.<sup>24,25</sup> The programming functions of these instruments also accept input regarding the starting and desired ending hematocrit of the patient, the average hematocrit of red cell replacement units to be used, and the desired fluid balance (a default of 100% may be offered by the instrument). Finally, the operator enters the desired fraction of the patient’s own red cells (fraction cells remaining or FCR) that should remain in the patient’s circulation at the end of the procedure. The instrument calculates the volume of replacement fluid (eg, red cells of average hematocrit as programmed into the instrument’s computer) needed for the procedure.<sup>24</sup>

Calculation of the desired FCR is predicated on the targeted therapeutic endpoint of the red cell exchange.<sup>21,23,26-30</sup> Thus (Formula 1):

$$\text{FCR (as \%)} = 100 \times \left( \frac{\text{starting hematocrit}}{\text{desired ending hematocrit}} \times \frac{\text{endpoint parameter}}{\text{starting parameter}} \right)$$

where the specified parameter may be the % hemoglobin S, the % parasitized red cells seen on the peripheral blood film, etc.

According to ASFA, red cell exchange is indicated (Category I or II) for treatment of severe manifestations of the protozoal infections *Plasmodium falciparum* and *Babesia microti* and for the management of sickle cell disease (SCD).<sup>1,6</sup> Therefore, if a patient with sickle cell anemia whose hematocrit is 27% and whose starting hemoglobin S level is 100% is to undergo red cell exchange, and if the targeted endpoint hemoglobin S level is 30% and the desired ending hematocrit is 30%, then the FCR is calculated accordingly:

$$\text{FCR (as \%)} = 100 \times \left( \frac{27\%}{30\%} \times \frac{30\%}{100\%} \right) = 27\%$$

At the end of the procedure, the patient's own red cells will amount to 27% of the circulating red cell mass.

### Sickle Cell Disease

Sickled erythrocytes were first described in 1910 by Dr. JB Herrick, who noted the abnormally shaped red cells in the peripheral blood film of a dental student from the Caribbean island of Grenada,<sup>31</sup> but the malady known now as sickle cell anemia was familiar to the people of central and west Africa.<sup>32,33</sup> Caused by homozygosity for the mutant  $\beta^S$  gene on chromosome 11,<sup>34-36</sup> the disease likely resulted in a survival disadvantage in Africa.<sup>37</sup> However, the gene frequency remained high because of the protection from *P. falciparum* malaria afforded to heterozygote children<sup>38</sup> and because of social factors.<sup>39</sup>

The substitution of valine for glutamic acid as the sixth amino acid residue from the amino terminus of the hemoglobin  $\beta$  chain results in hydrophobic interactions between nearby hemoglobin S molecules such that hemoglobin S aggregates into large polymers when deoxygenated.<sup>40</sup> The polymerization of hemoglobin S in vivo is the underlying basis for the hemolytic and vaso-occlusive morbidity of SCD,<sup>26,40,41</sup> and the exquisite dependence of polymerization on the concentration of hemoglobin S<sup>42,43</sup> has provided a scientific basis, in concert with the strong clinical basis,<sup>44</sup> for transfusion therapy in sickle cell anemia.

For the most part, clinical studies related to transfusion management of SCD have focused on simple transfusion or manual exchange transfusion.<sup>23,26,27,44-50</sup> The efficacy of manual vs automated red cell exchange in the treatment of SCD has not been directly studied in a clinical trial; however, red cell exchange can be completed more efficiently and quickly by automated than by manual methods.<sup>21</sup> Automated red cell exchange has been shown to mitigate iron overload, while maintaining a low hemoglobin S level, in SCD patients receiving chronic treatment.<sup>51-53</sup> Thus, it has entered into routine use in centers where therapeutic apheresis is available. Its role in the aspects of SCD for which it is indicated<sup>1,6</sup> is discussed below (see Table 43-1). Manual exchange transfusion, or automated red cell exchange using discontinuous flow equipment, may be performed using isotonic saline, rather than red cells, as the replacement fluid in the early phases of the procedure in order to maximize the removal of hemoglobin-S-containing red cells and avoid the gratuitous removal of normal red cells.<sup>23,51,53</sup>

### Life- or Organ-Threatening Complications

Red cell exchange is standard therapy for children with acute vaso-occlusive stroke (see below for discussion of stroke prevention) and should be performed shortly following documentation of thrombotic (rather than hemorrhagic) stroke by non-contrast computed tomography.<sup>21,23,44,54</sup> The treatment goal should be a hemoglobin concentration between 9 and 10 g/dL and less than 30% hemoglobin S. Acute intervention, followed by chronic maintenance transfusion therapy, may limit early morbidity and mortality and prevent recurrence (see below).<sup>23,27,44,46</sup>

Acute multiorgan failure syndrome is a complication of severe pain episodes of sickle cell disease.<sup>21,54,55</sup> It may present as an unusually severe pain episode in patients with sickle cell anemia or hemoglobin SC disease and is characterized by fever, accelerated hemolysis with a rapid decrease in hemoglobin and platelet count, nonfocal encephalopathy, and rhabdomyolysis.<sup>55</sup> Beside the central nervous system, other organs, including liver and kidney, may be involved.<sup>55</sup> Although standard red cell transfusion therapy may be effective if severe anemia is present, red cell exchange should be considered with higher hemoglobin levels.<sup>54</sup> This syndrome may be less likely in patients who are on aggressive chronic transfusion regimens in whom the frequency of episodes of pain or acute chest syndrome has been observed to be reduced.<sup>50</sup>

In its 2000 report<sup>49</sup> the National Acute Chest Syndrome Study Group (NACSSG) defined acute chest syndrome, the leading cause of death and hospitalization of patients with SCD,<sup>56,57</sup> on the basis of presentation with a new alveolar infiltrate involving one or more complete lung segments (atelectasis excluded) and accompanied by chest pain, a fever  $>38.5^\circ\text{C}$ , tachypnea, wheezing, or cough. Although the NACSSG report did not describe a particular advantage of red cell exchange over simple transfusion, the trial was not randomized for the purpose of detecting such a difference.<sup>21,49</sup> Many experienced practitioners recommend exchange transfusion for patients who present with diffuse pulmonary involvement or progressive respiratory decline despite simple transfusion, the goal being a hemoglobin no higher than 10 g/dL and less than 30% hemoglobin S.<sup>21,44,54</sup>

Although priapism has not been evaluated by ASFA as an indication for automated red cell exchange,<sup>1</sup> it occurs in approximately 30% to 90% of males with SCD<sup>58-60</sup> and thus may result in consultation requests to the apheresis service. Early claims of success in applying red cell exchange therapy to priapism after 12 to 24 hours of unresponsiveness to conventional treatments were unconvincing in that detumescence was rarely noted sooner than 24 to 48 hours after red cell exchange,

**Table 43-1.** Red Cell Exchange in Sickle Cell Disease

|                   |                                                                                                                                           |
|-------------------|-------------------------------------------------------------------------------------------------------------------------------------------|
| Acute or emergent | Acute vaso-occlusive stroke<br>Multiorgan failure syndrome<br>Acute chest syndrome refractory to standard management                      |
| Chronic           | Prevention of stroke in high-risk children<br>Secondary stroke prevention<br>Prevention of iron overload in chronic transfusion recipient |

and often not until 2 to 7 days after treatment, and there were no controlled studies.<sup>44,61</sup> A cautionary note regarding red cell exchange for the treatment of priapism was introduced with the description of acute, severe neurologic abnormalities in six boys with sickle cell anemia 1 to 11 days following partial exchange transfusion for priapism unresponsive to conservative therapy.<sup>62</sup> The syndrome was characterized by severe headache at the onset, often associated with increased intracranial pressure, and further neurologic events ranging from seizure activity to obtundation requiring ventilatory support.<sup>62,63</sup> The mean posttreatment hemoglobin of the affected children was 12.1 g/dL, and the syndrome, termed ASPEN (association of sickle cell disease, priapism, exchange transfusion and neurologic events), was attributed to cerebral ischemia after an acute rise in hemoglobin, concomitant decrease in hemoglobin S and release of vasoactive mediators during detumescence.<sup>62,63</sup> A recent series<sup>64</sup> described six adult men and one child with sickle cell anemia, six of whom received automated red cell exchange 28 to 48 hours after onset of an episode of priapism (one was treated 7 days after onset of priapism). All of them ultimately required surgical decompression procedures. Finally, a comprehensive review of 42 well-documented case reports of transfusion therapy in SCD-associated priapism evaluated the effectiveness of transfusion therapy vs conventional therapies in terms of time to detumescence.<sup>65</sup> The mean time to detumescence with transfusion therapies was 10.8 days (26 cases) vs 8.0 days with conventional therapies (16 cases). Neurologic complications with transfusion therapy were described in nine cases, some with persistent long-term deficits.

### Primary and Secondary Prevention of Stroke

Approximately 5% to 10% of untransfused children with SCD will have a cerebral infarction by age 20.<sup>45,66</sup> Chronic transfusion therapy, given every 3 to 4 weeks, to maintain the level of hemoglobin S below 30% can improve the arteriographic appearance of affected cerebral vessels and reduce the risk of recurrent stroke from 66% to 90% to approximately 10%.<sup>45,46,54</sup> Chronic automated red cell exchange can be substituted for simple transfusion.<sup>51-53</sup> Some authors argue in favor of discontinuation of chronic transfusion therapy after 3 or more years<sup>67</sup>; however, reports of recurrent stroke rates of 50% or greater after discontinuation of transfusion therapy have led most sickle cell treatment programs to recommend indefinite prophylactic transfusion regimens.<sup>27,54,68,69</sup>

The utility of chronic transfusion therapy to decrease the risk of recurrent stroke in children with SCD<sup>27,45,46,54</sup> and the demonstration that transcranial Doppler ultrasound was highly predictive of stroke risk in such children,<sup>70,71</sup> led to the Stroke Prevention Trial in Sickle Cell Anemia (STOP trial), which sought to study the ability of transfusion therapy to prevent a first stroke in high-risk children with SCD.<sup>48</sup> Time-averaged mean blood-flow velocity of at least 200 cm/sec in the internal carotid or middle cerebral artery<sup>70,71</sup> and a stroke-free history were required for study entry.<sup>48</sup> Over a period of approximately 2 to 3 years, transfusion therapy to maintain hemoglobin S below 30% without exceeding a hemoglobin concentration of 12 g/dL

reduced the occurrence of stroke in the treatment group by 90% compared to the control group.<sup>48</sup> A follow-up study (STOP 2)<sup>72</sup> examined the effect of discontinuation of transfusion therapy after 30 months in children from the first STOP trial whose transcranial Doppler readings had reverted to normal. The study was ended prematurely after 79 of an intended 100 subjects had been enrolled. An interim analysis revealed that among 41 children who had been randomly assigned to discontinue transfusions, 14 had reverted to high-risk transcranial Doppler findings 4 to 9 months after stopping transfusions and two had had ischemic strokes. No neurologic events or high-risk Doppler findings were noted in the subjects randomly assigned to continue transfusion therapy. The National Heart, Lung, and Blood Institute recommends continuing transfusion therapy beyond 30 months.<sup>73</sup>

### Transfusional Iron Overload

There are no randomized, prospective comparisons of simple transfusion vs automated red cell exchange in the prevention of iron overload in children with SCD who require chronic transfusion therapy. However, four case series of 8 to 14 subjects<sup>51-53,74</sup> have been reported in which children were either converted from a simple transfusion program to monthly red cell exchange or begun on red cell exchange early on. In addition, some in each series were on chelation therapy with desferrioxamine<sup>23</sup> and some were not. In general, red cell exchange resulted in a 25% to 100% increase in red cell usage and a corresponding variable increase in donor exposures. However, serum ferritin tended to stabilize in those who were not on chelation therapy and significantly decrease in those who continued on chelation therapy. Some children who were begun on red cell exchange before development of iron overload did not accumulate iron as a result of their red cell exchange treatments.

### Protozoan Disease

Severe manifestations of malaria and babesiosis are ranked as Category II indications by ASFA, largely on the basis of anecdotal evidence.<sup>1,2</sup>

### Malaria

Severe malaria is caused by infection with *P. falciparum*.<sup>75</sup> The infection results from injection of sporozoites into the bloodstream by the bite of a female *Anopheles* mosquito, the division of the malarial sporozoites into merozoites in the liver, and the development of the merozoites into ring-form trophozoites, and then schizonts, within invaded erythrocytes in the circulation.<sup>75</sup> These asexual blood stages of *P. falciparum* are responsible for the symptoms of severe malaria that occur when infection is complicated by serious organ failure or metabolic dysfunction (see Table 43-2).<sup>75-79</sup>

The manifestations of severe *P. falciparum* malaria appear to derive from the adherence of infected erythrocytes to glycosylated molecules on endothelium and to platelet CD36 via *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1), an adhesive protein encoded by the parasite genome and expressed on the surface of infected erythrocytes.<sup>80,81</sup> The sequestration of parasitized

**Table 43-2.** Severe Clinical Manifestations of *Plasmodium falciparum* Malaria

|                         |                                                                                                                                                |
|-------------------------|------------------------------------------------------------------------------------------------------------------------------------------------|
| General                 | Prostration or weakness                                                                                                                        |
| Cerebral malaria        | Impaired consciousness<br>Abnormal behavior<br>Seizures ( $\geq 3$ per 24 hours)<br>Coma (persists at least 30 minutes after seizure)          |
| Severe anemia           | Hemolysis<br>Hemoglobin $< 5$ mg/dL or hematocrit $< 15\%$<br>Bilirubin $> 2.5$ mg/dL<br>Macroscopic hemoglobinuria not due to G6PD deficiency |
| Fever                   | Core body temperature $> 40^{\circ}\text{C}$ ( $104^{\circ}\text{F}$ )                                                                         |
| Renal failure           | Creatinine $> 3.0$ mg/dL<br>Urine output $< 400$ mL/24 hours in adults                                                                         |
| Pulmonary abnormalities | Pulmonary edema<br>Acute respiratory distress syndrome<br>Hypoxemia                                                                            |
| Hypoglycemia            | Blood glucose $< 40$ mg/dL                                                                                                                     |
| Shock                   | Systolic blood pressure 70 mm Hg                                                                                                               |
| Hemostatic defects      | Clinical and/or laboratory evidence of disseminated intravascular coagulation                                                                  |
| Metabolic acidosis      | Arterial pH $< 7.25$<br>Plasma bicarbonate $< 15$ mmol/L                                                                                       |
| Hyperparasitemia        | $> 5\%$ of erythrocytes parasitized                                                                                                            |

erythrocytes in endothelium in the brain and other organs results in vascular obstruction and elaboration of proinflammatory cytokines such as tumor necrosis factor- $\alpha$  and interferon- $\gamma$  that, in addition to other factors, may result in the severe manifestations of the disease.<sup>80,81</sup> Thus, the use of exchange transfusion and automated red cell exchange in the treatment of severe malaria is based on an ability to rapidly reduce the burden of parasitemia and the potential to thereby improve the rheologic properties of the blood and reduce the level of toxic mediators such as cytokines.<sup>29</sup> Red cell exchange followed by plasma exchange, or automated whole blood exchange, has been described as an approach to the removal of both parasitized red cells and inflammatory mediators in the treatment of severe *P. falciparum* malaria. However, the evidence to support this approach is anecdotal at best.<sup>29</sup>

A meta-analysis of eight case-control studies did not find a survival advantage in using exchange transfusion as an adjunct to the treatment of severe *P. falciparum* malaria<sup>82</sup>; however, the authors speculated that automated red cell exchange might be a promising alternative (the studies analyzed employed manual whole blood exchange transfusion). Five case series (16 patients total) and one case report describe lowering of parasitemia by 80% to 90% using automated red cell exchange of 1.0 to 1.5 red cell volumes in approximately 2 hours, followed by rapid clinical recovery, in cases of severe *P. falciparum* malaria, including cerebral malaria.<sup>82-87</sup> The United States Centers for Disease

Control and Prevention (CDC) recommends strong consideration of exchange transfusion or automated red cell exchange if parasite density is  $> 10\%$  or in case of complications such as cerebral malaria, acute respiratory distress syndrome (ARDS), or renal failure.<sup>88</sup> Treatment may be repeated until parasitemia involves  $< 1\%$  of circulating red cells.<sup>29,88</sup>

### Babesiosis

Babesiosis in humans is a zoonotic disease and is spread to humans primarily through ticks of the genus *Ixodes*.<sup>89</sup> In the Northeastern United States, where most cases of human babesiosis occur, the predominant organism is *Babesia microti*, the reservoir hosts are wild rodents, and the vector for transferring the protozoa from host to humans is the deer tick *Ixodes dammini*, the same tick that transmits *Borrelia burgdorferi*, causative agent of Lyme disease.<sup>89,90</sup> The first two reported cases of human babesiosis, one from Europe<sup>91</sup> and one from California,<sup>92</sup> occurred in splenectomized individuals. The first case in a patient with an intact spleen was reported in Nantucket.<sup>93</sup>

When injected into the human bloodstream, the sporozoites of babesia are presumed to complex with C3 in the circulation and then enter erythrocytes by attaching to the C3b receptor.<sup>30</sup> After asexual budding into four merozoites the parasite perforates the erythrocyte membrane, resulting in hemolysis. They are then free to infect other erythrocytes, and transform into dividing trophozoites (ring forms and tetrads visible on peripheral blood films).<sup>30</sup>

After an incubation period of 1 to 6 weeks following inoculation, infected patients develop a flu-like syndrome characterized by fever, fatigue, and malaise.<sup>30,90</sup> Headache, chills, sweats, myalgia, and arthralgia are frequent complaints. Physical findings may include fever and splenomegaly, and jaundice and pallor may accompany marked extravascular hemolysis.<sup>94</sup> Although most cases are subclinical or mild,<sup>94,95</sup> severe manifestations, including disseminated intravascular coagulation, respiratory failure, and renal failure may occur.<sup>28</sup> Immunocompromised or asplenic individuals are typically more severely affected.<sup>96</sup>

Although clinical trials are lacking, several case reports and case series suggest that, given the absence of an exoerythrocytic phase of infection, red cell exchange, whole blood exchange or red cell exchange followed by plasma exchange, combined with antibiotic therapy, can be beneficial in severe cases of babesiosis with  $> 5\%$  parasitemia.<sup>6,28,29,97</sup> A single one- to two-volume red cell exchange can reduce the circulating population of parasitized erythrocytes by 85% to 90%.<sup>6</sup>

### Erythrocytapheresis and Therapeutic Phlebotomy (Venesection)

According to ASFA, symptomatic erythrocytosis and polycythemia vera, clinical entities that are characterized by an elevated circulating total red cell volume,<sup>98,99</sup> are Category II indications for red cell volume reduction by erythrocytapheresis.<sup>1,6</sup> In clinical practice, therapeutic phlebotomy, rather than automated erythrocytapheresis, is a mainstay of management.<sup>100,101</sup> The International Council for Standardization in

**Table 43-3.** Diagnostic Criteria for Polycythemia Vera

| Category  | Polycythemia Vera Study Group (US) <sup>102</sup>              | Pearson and Messinezy (British) <sup>114</sup>                                                              | World Health Organization <sup>115</sup>                                                                                                      |
|-----------|----------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------|
| A         | 1. Total RCV<br>Male $\geq 36$ mL/kg<br>Female $\geq 32$ mL/kg | 1. Total RCV $>25\%$ above normal predicted mean                                                            | 1. Total RCV $>25\%$ above normal predicted mean or hemoglobin $>99$ th percentile of reference for age, gender, altitude                     |
|           | 2. Arterial O <sub>2</sub> sat $\geq 92\%$                     | 2. Absent cause of secondary erythrocytosis                                                                 | 2. Absent cause of secondary erythrocytosis:<br>Familial<br>O <sub>2</sub> sat $\leq 92\%$<br>High-affinity hemoglobin<br>EPO-producing tumor |
|           | 3. Splenomegaly                                                | 3. Palpable splenomegaly                                                                                    | 3. Splenomegaly                                                                                                                               |
| B         | 1. Platelets $>400,000/\mu\text{L}$                            | 4. Abnormal marrow karyotype                                                                                | 4. Abnormal marrow karyotype (not bcr-abl)                                                                                                    |
|           | 2. White cells $>12,000/\mu\text{L}$                           | 1. Platelets $>400,000/\mu\text{L}$                                                                         | 1. Platelets $>400,000/\mu\text{L}$                                                                                                           |
|           | 3. LAP $>100$                                                  | 2. Neutrophils $>10,000/\mu\text{L}$ (12,500/ $\mu\text{L}$ in smokers)                                     | 2. WBC $>12,000/\mu\text{L}$                                                                                                                  |
|           | 4. Serum B <sub>12</sub> $>900$ pg/mL                          | 3. Splenomegaly (by imaging study)                                                                          | 3. Marrow biopsy reveals panmyelosis                                                                                                          |
| Diagnosis | A1 + A2 + A3 or<br>A1 + A2 + any 2 B                           | 4. EPO-independent BFU-E growth in vitro, or low serum EPO value<br>A1 + A2 + A3 or A4<br>A1 + A2 + any 2 B | 4. Low serum EPO levels<br>A1 + A2 + any other A or 2 B                                                                                       |

RCV = red cell volume ("red cell mass"); LAP = leukocyte alkaline phosphatase; EPO = erythropoietin; BFU-E = burst-forming unit erythroid, a marrow-derived erythroid colony in culture.

Haematology recognizes absolute erythrocytosis in an individual whose measured total red cell volume (often referred to as "red cell mass"<sup>102</sup>) is more than 25% above the mean predicted value for a person of the same body surface area.<sup>98</sup> This will be the case in men with a hematocrit of 60% or higher and women with a hematocrit of 56% or higher.<sup>98</sup>

### Polycythemia Vera

Polycythemia vera has been acknowledged as a distinct clinical disorder for more than a century.<sup>103,104</sup> Classified as a bcr/abl-negative myeloproliferative disorder, polycythemia vera is characterized by panmyelosis in the marrow and peripheral blood, splenomegaly, hyperviscosity of the blood, thrombosis and a tendency to evolve into either acute myeloid leukemia or myelofibrosis.<sup>99,101,105,106</sup> Over 95% of cases are now known to be associated with an acquired point mutation, V617F, in exon 14 of the protein tyrosine kinase Janus kinase 2 (JAK2).<sup>107-111</sup> The remaining 5% of patients appear to have mutations in exon 12 of JAK2, including some who may have a more benign phenotype (idiopathic erythrocytosis).<sup>112,113</sup> Popular

diagnostic systems for polycythemia vera are presented in Table 43-3.<sup>102,114,115</sup> A revised diagnostic system that recognizes the predominance of JAK2 mutations in polycythemia vera has been proposed.<sup>116</sup>

### Therapeutic Phlebotomy in Polycythemia Vera

The increased whole blood viscosity that results from the expansion of total red cell volume in patients with polycythemia vera is the underlying basis of the life-threatening prothrombotic state and the headache, fatigue, dyspnea, cyanosis, and other signs and symptoms that characterize the disorder.<sup>101,105</sup> Aggressive phlebotomy to a hematocrit below 45% in males of European ancestry and below 42% in females of European ancestry and in both genders of African ancestry is indicated for prevention of life-threatening thrombotic complications of polycythemia vera.<sup>101,105,117</sup> High-risk patients (age  $>65$  years or history of cardiovascular risk factors such as hypertension, diabetes, hypercholesterolemia, congestive heart failure, smoking, or thrombosis) should also be treated with cytoreductive chemotherapy (eg, hydroxyurea, busulfan, interferon- $\gamma$ ).<sup>101,117</sup> Low-risk patients (age

<60 years, no cardiovascular risk, platelet count <1,500,000/ $\mu$ L) may initially be managed with phlebotomy alone.<sup>101,117</sup> Low-dose aspirin (100 mg/day) is recommended for all patients without specific contraindications to its use.<sup>117,118</sup> The pharmaceutical development of JAK2-selective kinase inhibiting agents may influence future management of polycythemia vera.<sup>119</sup>

### Erythrocytapheresis in Polycythemia Vera

A retrospective case series<sup>120</sup> of 69 patients with polycythemia vera who underwent 206 isovolemic erythrocytapheresis procedures using 4% albumin as replacement fluid<sup>121</sup> reported reduction of hematocrit from  $56.8 \pm 5.6\%$  to  $41.9 \pm 6.6\%$  after removal of  $1410 \pm 418$  mL of red cells with a hematocrit of  $79.7 \pm 9.3\%$ .<sup>120</sup> A subset of 21 patients for whom close follow-up data were available, and whose hematocrit was reduced from  $58 \pm 5.7\%$  to  $41.5 \pm 4.9\%$  by a single erythrocytapheresis procedure maintained a hematocrit of <50% for a median of 6 months.<sup>120</sup> The durability of response to a single procedure was associated with a median 70% inhibition of in-vitro erythropoietin-independent erythroid burst-forming unit growth that the authors attributed to iron removal during the apheresis procedure.<sup>122</sup> This claim has not been confirmed. Automated erythrocytapheresis may be useful in polycythemia vera for the rapid induction of hematocrit lowering, followed by maintenance therapeutic phlebotomy, for emergent isovolemic hematocrit lowering in patients with acute thrombotic or microvascular complications, or to avoid perioperative thrombohemorrhagic complications in a patient with an uncontrolled hematocrit who requires urgent surgery.<sup>6,121</sup>

The volume of red cells to be removed (VR) during an erythrocytapheresis in order to achieve a desired hematocrit can be calculated as (Formula 2)<sup>121</sup>:

$$VR = \left[ \frac{(\text{starting hematocrit} - \text{desired hematocrit})}{79} \right] \times [\text{blood volume (mL/kg)}] \times [\text{body weight (kg)}]$$

Thus, for a 70-kg person with a blood volume of 70 mL/kg whose hematocrit is to be lowered from 68% to 55%, the volume of red cells to be removed is calculated as:

$$VR = [(68 - 55) \div 79] \times 70 \times 70 = 910 \text{ mL of red cells.}$$

Formula 2 can be used to estimate the volume of replacement fluid (crystalloid or colloid) needed to maintain fluid balance.<sup>121</sup> An easy alternative is to program an automated apheresis instrument to perform a “red cell exchange” using replacement fluid with hematocrit of 0%, a target FCR of 100%, and a target hematocrit as desired (see Formula 1).

### Secondary Erythrocytosis

Secondary erythrocytosis refers to conditions that result in an elevated total red cell volume but are not clonal disorders of the marrow.<sup>98,99,101,102,123</sup> Congenital and acquired causes have been

**Table 43-4.** Secondary Erythrocytosis

| Type       | Underlying Cause                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
|------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Congenital | High oxygen-affinity hemoglobin <sup>124</sup><br>Bisphosphoglycerate mutase deficiency <sup>125</sup><br>Chuvash polycythemia <sup>126,127</sup><br>Erythropoietin receptor mutation <sup>128</sup>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |
| Acquired   | Hypoxia-stimulated <sup>98,99,101,123</sup> <ul style="list-style-type: none"> <li>• Cyanotic congenital heart disease</li> <li>• Chronic lung disease</li> <li>• High-altitude habitat</li> <li>• Smoker's erythrocytosis</li> <li>• Carbon monoxide poisoning</li> <li>• Chronic hypoventilation (sleep apnea)</li> <li>• Renal artery stenosis</li> </ul> Inappropriate erythropoietin production <sup>98,99,101,123</sup> <ul style="list-style-type: none"> <li>• Renal cancer</li> <li>• Hepatic cancer</li> <li>• Cerebellar hemangioblastoma</li> <li>• Endocrine tumors</li> <li>• Uterine leiomyoma</li> <li>• Polycystic kidney</li> <li>• Meningioma</li> </ul> Drug-mediated <sup>98,99,101,123</sup> <ul style="list-style-type: none"> <li>• Androgen therapy</li> <li>• “Blood doping” (surreptitious erythropoietin use)</li> </ul> Multifactorial etiology <ul style="list-style-type: none"> <li>• Postrenal transplant erythrocytosis<sup>129</sup></li> </ul> |

described, and predominantly involve the regulation or aberrant expression of erythropoietin or abnormalities of the erythropoietin receptor (see Table 43-4).<sup>98,99,101,102,123-129</sup> A diagnostic investigation of a patient with suspected erythrocytosis is performed in order to 1) establish that a true state of erythrocytosis exists (eg, an elevated total red cell volume), 2) distinguish secondary erythrocytosis from polycythemia vera, and 3) determine the cause of secondary erythrocytosis, thereby guiding the approach to clinical management.<sup>101,102,116,123,130,131</sup>

### Therapeutic Phlebotomy in Secondary Erythrocytosis

The role of phlebotomy is less certain in secondary erythrocytosis than in polycythemia vera.<sup>123</sup> As suggested by Table 43-4, secondary erythrocytosis is generally an adaptation to the disordered regulation of erythropoietin or to hypoxemia. In some cases the underlying cause can be treated medically or surgically, and in others the erythrocytosis represents a physiologic adaptation to a chronic condition. For example, adults with cyanotic congenital heart disease are not considered to be at heightened risk for thrombotic stroke despite mean hematocrits of  $57.5\% \pm 7.2\%$ <sup>132</sup> and do not exhibit symptoms of hyperviscosity at hematocrits below 65% in the absence of dehydration or iron deficiency.<sup>133</sup> In fact, microcytosis or a history of phlebotomy are significant risk factors for stroke in this population, as are atrial fibrillation and hypertension.<sup>134</sup>

Isovolemic phlebotomy, with saline replacement, should be reserved for patients with moderate symptoms of hyperviscosity (ie, headache, slow mentation, visual disturbance, tinnitus, dizziness, etc), and who are neither dehydrated nor iron deficient.<sup>133</sup> A program of therapeutic phlebotomy should not be undertaken purely for the sake of achieving a target hematocrit in an asymptomatic individual. Withdrawal of up to a unit of whole blood, replaced by 750 to 1000 mL of isotonic saline, has been recommended for relief of symptoms.<sup>133</sup> Preoperative autologous blood donation if the hematocrit is above 65% may also be considered.<sup>133</sup> Similar recommendations may refer to patients with high oxygen-affinity hemoglobin levels who have symptoms such as dizziness, dyspnea, or angina, which are believed to result, in part, from an expanded total red cell volume.<sup>101,123</sup> There is no formal evidence that phlebotomy is beneficial, and a modest target (ie, a hematocrit <60% achieved by partial exchange transfusion) has been recommended.<sup>101,123</sup> Patients with chronic hypoxic lung disease and erythrocytosis or with smoker's erythrocytosis are best managed using medical therapy to deal with their underlying pulmonary disorder. Non-controlled studies suggest that phlebotomy to a hematocrit of 50% to 52% may improve exercise tolerance, alleviate headache and confusion, and otherwise ameliorate symptoms of hyperviscosity.<sup>101,123</sup>

Postrenal transplant erythrocytosis, defined as a hematocrit above 51% to 55%, occurs spontaneously in 15% to 20% of kidney transplant recipients in the first 8 to 24 months after engraftment.<sup>129,135-137</sup> One fourth of cases remit spontaneously within 2 years of onset, the balance persisting for up to several years until chronic graft rejection supervenes.<sup>123</sup> Male gender, retention of native kidneys, smoking, a rejection-free course with a well-functioning graft, and limited or no need for erythropoietin during the period of pretransplant hemodialysis have been listed as risk factors for postrenal transplant erythrocytosis.<sup>129,135</sup> Hyperviscosity symptoms such as malaise, headache, plethora, lethargy, and dizziness are described as common among patients with this condition, and 10% to 30% develop significant thromboembolic complications.<sup>129,135</sup> The pathogenesis appears to be multifactorial, and likely involves an interplay between endogenous erythropoietin production by the retained native kidney, the renin-angiotensin system, androgen secretion, insulin-like growth factors, and cytokines.<sup>129,136,137</sup> One retrospective series reported 11 thromboembolic events including transient ischemic attacks and strokes, and venous thromboembolism in 10 of 53 (19%) patients with postrenal transplant erythrocytosis but in none of 49 control cases ( $p < 0.001$ ).<sup>135</sup> This sort of experience has led to an appreciation of the need to control the red cell volume in these patients.<sup>129,135-137</sup> The mainstay of treatment is angiotensin-converting enzyme inhibition or angiotensin-converting enzyme receptor blockade, sometimes in combination with theophylline, which lowers hemoglobin and hematocrit within 8 weeks with peak effect seen after up to 12 months.<sup>138-142</sup> Cautious phlebotomy may be used initially to lower the hematocrit to 45% to 50%; however, using

phlebotomy to maintain a target hematocrit over time results in iron deficiency.<sup>129,142</sup>

### ***Erythrocytapheresis in Secondary Erythrocytosis***

Although automated erythrocytapheresis is seldom recommended for management of secondary erythrocytosis,<sup>121</sup> it may be useful in circumstances where isovolemic procedures are called for, such as in cyanotic heart disease.<sup>133</sup>

One retrospective series reported 208 erythrocytapheresis procedures involving 61 patients, most of whom appeared to have chronic pulmonary insufficiency or cyanotic congenital heart disease (details were not provided).<sup>143</sup> The volume of whole blood processed was  $1322 \pm 446$  mL in two to four cycles (discontinuous flow equipment) at a whole blood flow rate of 60 mL/minute, a mean of 576 mL (range 426-800 mL) of red cells was removed per procedure, mean hematocrit was reduced from  $58.4\% \pm 4.5\%$  to  $42.7\% \pm 5.3\%$ , and patients were maintained with a hematocrit below 50% for an average of 6.5 months after every procedure.<sup>143</sup> Another descriptive case series claimed that hemoglobin, hematocrit, and blood viscosity could be lowered "drastically" for as long as 11 months by a single erythrocytapheresis procedure.<sup>14</sup> Apheresis has not been reported in the management of posttransplant erythrocytosis.

### ***Hereditary Hemochromatosis***

Although not covered in the 2007 ASFA review of indications for therapeutic apheresis,<sup>1,6</sup> hereditary hemochromatosis is an important indication for therapeutic phlebotomy<sup>144</sup> and erythrocytapheresis has been described as an alternative to phlebotomy in its management.<sup>145</sup> This is an inherited disorder that, untreated, results in iron deposition in, and damage to, the liver, heart, pancreas, and other organs.<sup>144,146,147</sup> Its prevalence is approximately 1:200 among those of European ancestry.<sup>148-150</sup> The most common genetic mutation, accounting for >90% of cases (and almost all cases in persons of Northern European ancestry) is homozygosity for a single missense mutation (G to A) at nucleotide 845 in the HFE gene on chromosome 6p21.<sup>149-153</sup> This mutation results in substitution of cysteine with tyrosine at amino acid 282 and is referred to as the C282Y mutation.<sup>154</sup> Abnormalities of the HFE gene may result in a defect in iron sensing in the deep crypt cells of gut epithelium and thus inappropriate iron uptake despite abundant iron stores in the body.<sup>149</sup> The accumulation of iron in the liver and other organs slowly results in liver failure (cirrhosis, hepatocellular carcinoma), diabetes, hypogonadism, hypopituitarism, arthropathy, cardiomyopathy and heart failure, and skin pigmentation.<sup>149,152</sup> A presenting syndrome of asthenia, arthralgia, and abnormal liver function has been described as classic for the clinical disease.<sup>147</sup> Because of the central importance of iron loading in the pathogenesis of hereditary hemochromatosis, iron removal by therapeutic phlebotomy is the mainstay of treatment.<sup>155</sup> Additional genetic associations with hereditary hemochromatosis, and diagnostic elements of the condition, are provided in Tables 43-5 and 43-6.

**Therapeutic Phlebotomy in Hereditary Hemochromatosis**

Therapeutic phlebotomy has been the primary mode of iron reduction in hereditary hemochromatosis for over a half century.<sup>144,155,160</sup> Phlebotomy therapy should be started in all patients whose serum ferritin level is elevated (see Table 43-6) and should not be withheld from the elderly on the basis of age or from iron-loaded patients who have not developed clinical symptoms.<sup>144,155</sup> A common treatment approach is to perform one phlebotomy per week (1 unit or 7 mL/kg of whole blood not to exceed 550 mL per phlebotomy) until the serum ferritin is  $\leq 50$  ng/mL.<sup>144,155</sup> Thereafter, it is usually necessary to annually remove 3 to 4 units of blood to maintain the ferritin at that level. Malaise, weakness, fatigability, and liver transaminase elevations often improve during the first several weeks of treatment, but joint symptoms may initially worsen before eventually improving (if at all).<sup>144,155</sup> Cardiomyopathy and cardiac arrhythmias may resolve with phlebotomy, but insulin-dependent diabetes generally will not. The risk of hepatocellular carcinoma will persist if cirrhosis was present before the onset of phlebotomy therapy.<sup>144,155</sup> Hypogonadotropic hypogonadism and thyroid dysfunction do not usually improve with phlebotomy; however, pituitary and gonadal function may improve if they were of recent onset at the time phlebotomy therapy was started.<sup>144</sup>

**Table 43-5.** Genetic Mutations Resulting in Hereditary Hemochromatosis Syndromes<sup>156,157</sup>

| Type               | Gene                               | Chromosome | Mutation                                                                                                                                                                       |
|--------------------|------------------------------------|------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| I*<br>(classical)  | <i>HFE</i>                         | 6p21       | Cys282→Tyr (C282Y)<br>His63→Asp (H63D)<br>Ser65→Cys (S65C)                                                                                                                     |
| IIa*<br>(juvenile) | <i>HJV</i><br>(hemojuvelin)        | 1q21       | Gly320→Val (G320V)                                                                                                                                                             |
| IIb*<br>(juvenile) | <i>HAMP</i> (hepcidin)             | 19q13      | 93delG<br>Arg56→Ter (R56X)<br>Gly→71Asp (G71D)<br>G-A, +14 5-prime UTR                                                                                                         |
| III*               | <i>TFR2</i> (transferrin receptor) | 7q22       | Tyr250→Ter (Y250X)<br>Glu60→Ter (E60X)<br>Met172→Lys (M172K)<br>Gln690→Pro (Q690P)                                                                                             |
| IV†                | <i>SLC40A1</i><br>(ferroportin)    | 2q32       | Asn144→His<br>Ala77→Asp (A77D)<br>Val162 del<br>Asp157→Gly (D157G)<br>Gln182→His (Q182H)<br>Gly323→Val (G323V)<br>Asp181→Val (D181V)<br>Gly80→Val (G80V)<br>Gly267→Asp (G267D) |

\*autosomal recessive

†autosomal dominant

**Erythrocytapheresis in Hereditary Hemochromatosis**

Early reports from Europe<sup>14,15,17,161</sup> suggested that automated erythrocytapheresis could efficiently remove red cells and deplete iron in patients with hemochromatosis. A German group from Munich reported successful lowering of iron in 14 patients with hemochromatosis, with intervals of 2 to 11 months between procedures.<sup>14</sup> An Italian group<sup>15</sup> treated 14 patients with a mean of 93 (range 21-203) erythrocytapheresis procedures over a mean (median) of 9.6 (24) months. A mean of 19 (range 4.2-40.6) grams of iron were removed, transferrin saturation was reduced from  $90 \pm 8.7\%$  to  $17 \pm 10.6\%$ , serum ferritin decreased from  $3164 \pm 1488$  ng/mL to  $60.5 \pm 77.5$  ng/mL, and hepatic iron was said to have normalized in all cases. A follow-up study from Munich<sup>17</sup> described prospective observations on eight patients with hereditary hemochromatosis who were treated with isovolemic erythrocytapheresis (1000 mL removed) every 4 weeks until serum ferritin fell below 300 ng/mL. Iron depletion, thus defined, was achieved after a mean of 8.5 months during which a mean of 9.4 liters of red cells were removed in a mean of 8.9 procedures. Serum ferritin levels decreased from  $2596 \pm 399$  ng/mL to  $168 \pm 83$  ng/mL and transferrin saturation fell from  $91 \pm 6\%$  to  $19 \pm 10\%$ . Maintenance erythrocytapheresis every 5 to 6 months prevented iron reaccumulation during 18 to 36 months of follow-up.<sup>17</sup> A group from Zaragoza, Spain<sup>162</sup> compared data from nine patients with hemochromatosis who were treated with erythrocytapheresis over a period of 2 years to nine similar patients who were treated with conventional phlebotomy therapy. The erythrocytapheresis procedures were performed using a discontinuous-flow blood processor via single-needle access. A mean of 275 mL of red cells were removed with each procedure. The median serum ferritin reduction with each erythrocytapheresis was 55 ng/mL compared to 17 ng/mL

**Table 43-6.** Some Diagnostic Considerations in Hereditary Hemochromatosis (absent a cause of secondary iron overload)<sup>147,148,155,158,159</sup>

| Diagnostic Tool    | Factors to Consider                                                                                                                                                                                                                                                                                                                                                      |
|--------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Clinical clues     | <ul style="list-style-type: none"> <li>• Celtic ethnicity</li> <li>• Chronic asthenia</li> <li>• Arthropathy (fourth and fifth metacarpophalangeal joints: "hemochromatosis handshake")</li> <li>• Impotence</li> <li>• Hyperpigmentation</li> <li>• Liver abnormalities (transaminase elevation, hepatomegaly)</li> <li>• Diabetes</li> <li>• Cardiomyopathy</li> </ul> |
| Screening criteria | <ul style="list-style-type: none"> <li>• Transferrin saturation <math>\geq 50\%</math> in men; <math>\geq 45\%</math> in women</li> <li>• Serum ferritin: <math>\geq 200</math> ng/mL in premenopausal women; <math>\geq 300</math> ng/mL in men</li> </ul>                                                                                                              |
| Diagnostic tests   | <ul style="list-style-type: none"> <li>• Hepatic iron index <math>&gt; 1.9</math></li> <li>• Hepatic iron concentration <math>&gt; 80</math> <math>\mu</math>mol/g dry weight</li> <li>• Grade 3-4 hepatic iron deposition</li> <li>• HFE gene analysis</li> </ul>                                                                                                       |



with phlebotomy. Patients treated with erythrocytapheresis achieved their iron depletion goal in one third the time required for those treated with phlebotomy.<sup>162</sup>

A prospective evaluation of erythrocytapheresis in 13 patients with hereditary hemochromatosis was reported from Palma de Mallorca, Spain.<sup>145</sup> The volume of red cells to remove was calculated according to the patient's blood volume and preprocedure hematocrit (as noted in Formula 2 above), and the goal of each procedure was to remove a maximum of 800 mL of red cells and reduce the patient's hematocrit to 30%. Procedures were scheduled every 2 weeks, but the treatment interval would be extended for another 2 weeks if the starting hematocrit was found to be <36%. Starting serum ferritin was (mean ± SD) 1517 ± 1329 ng/mL, and was reduced to 20 ± 6.5 ng/mL after 6.7 ± 2.9 months and 13.5 ± 7.2 apheresis sessions. A mean of 565.5 ± 152 mL of red cells was removed with each procedure; this translated into removal of 878 ± 315 mg of iron per month. Four of the 13 patients underwent a maintenance erythrocytapheresis procedure after 6, 14, 16, and 16.5 months.<sup>145</sup>

A report from Buenos Aires<sup>163</sup> described a curious regimen of isovolemic erythrocytapheresis in combination with recombinant human erythropoietin, 4000 units subcutaneously three times per week (approximately 150 µg/kg/week) and folic acid 10 mg daily by mouth, in nonanemic patients with hereditary hemochromatosis. Ten men with hereditary hemochromatosis were subjected to erythrocytapheresis when their hematocrit reached 30% (or hemoglobin 10 g/dL), and the volume of red cells collected was calculated using Formula 2 as described above but in no case was greater than 30% of the circulating red cell mass. Procedures were performed with both continuous- and discontinuous-flow equipment using a three-phase apheresis protocol: collection of the red cell product, infusion of isotonic saline to replace the removed red cell volume, return of autologous plasma and buffy coat cells. A median of 3.1 erythrocytapheresis procedures (range 2.4-4.4) were performed each month during which a median of 2098 mL (1648-3677) of red cells were collected. It took a median of 9 (3-14) days for the hematocrit to reach 34% (or hemoglobin to rise to 10 g/dL) between procedures. Serum ferritin was reduced from a mean (range) of 2960 (1057-7200) ng/mL to 270 (184-398) ng/mL in a median of 3.25 (1.0-7.5) months.<sup>163</sup>

More recently, a pilot study from the Netherlands<sup>164</sup> reported results from six patients with hereditary hemochromatosis who received a mean (range) of 9.8 (5-18) erythrocytapheresis procedures over 4.8 (2-9) months using discontinuous-flow equipment. An estimated mean (range) of 465.2 (360-579) mg of iron were removed per procedure and a mean total of 4.38 (2.69-7.97) grams of iron were removed. Initial serum ferritin was 1419 (798-2541) ng/mL and final ferritin was 54 (39-90) ng/mL.<sup>163a</sup>

In summary, erythrocytapheresis in the treatment of hereditary hemochromatosis is supported by Type II-3 evidence<sup>1</sup> and may be an acceptable alternative to conventional schedules of

whole blood phlebotomy in selected patients. This indication has not yet been reviewed by ASFA and thus has not been assigned an indication category as of this writing.<sup>1</sup>

Therapeutic Platelet Apheresis

Thrombocytapheresis is a term that describes the selective removal of platelets from a patient for therapeutic purposes, using a blood processing (apheresis) device.<sup>1</sup> The 2007 ASFA review of indications for apheresis therapy lists *symptomatic thrombocytosis* as a Category II indication for thrombocytapheresis.<sup>1,6</sup> Although not made clear without a careful reading of the text,<sup>6</sup> this designation refers to primary thrombocytosis, as results from a clonal (myeloproliferative) disorder of the marrow.<sup>164</sup> Thrombocytapheresis for prophylaxis in asymptomatic patients or to lower the platelet count in cases of secondary or reactive thrombocytosis<sup>164</sup> is listed as a Category III (ie, unproven) indication because data either conflict or are insufficient to demonstrate benefit in these circumstances.<sup>1,6</sup> Some prominent causes of primary and secondary thrombocytosis are listed in Table 43-7.<sup>164-166</sup>

Secondary thrombocytosis per se does not convey a risk of thromboembolic morbidity absent confounding factors such as malignancy or major surgery.<sup>165,167</sup> Even then, antiplatelet agents should be the first option for treatment.<sup>166</sup> In any case, treatment of the underlying cause is the prime factor in the resolution of secondary thrombocytosis.<sup>164</sup> In fact, given the absence of risk posed by the platelet count in secondary thrombocytosis, the platelet count may be considered a laboratory sign of an underlying condition that should be investigated.<sup>164</sup>

Table 43-7. Some Causes of Primary (Clonal) and Secondary (Reactive) Thrombocytosis<sup>164-166</sup>

| Type                     | Possible Causes                                                                                                                                                                                                                                                        |
|--------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Primary thrombocytosis   | Essential thrombocytosis<br>Polycythemia vera<br>Chronic myelogenous leukemia<br>Myelofibrosis<br>Myelodysplastic syndrome (5q-)                                                                                                                                       |
| Secondary thrombocytosis | Acute hemorrhage<br>Chronic blood loss with iron deficiency<br>Tissue damage or trauma<br>Malignancy<br>Acute or chronic inflammation<br>Acute or chronic infection<br>Physical exercise<br>Rebound from chemotherapy or immune thrombocytopenic purpura<br>Medication |

### Consequences of Thrombocytosis in Primary Thrombocytosis (Essential Thrombocythemia)

Thrombocytosis with thromboembolic complications is a feature common to all of the chronic myeloproliferative disorders,<sup>168,169</sup> but essential thrombocythemia is the one most likely to be diagnosed in a patient who presents with thrombocytosis.<sup>165</sup> Thus, the remainder of this discussion will focus on essential thrombocythemia. Both thrombotic and hemorrhagic complications result in patients with essential thrombocythemia.<sup>164,170</sup> Factors that increase risk of thrombosis by threefold or more include age greater than 60 years and cardiovascular risk factors such as hypertension, hypercholesterolemia, and a prior thrombotic event.<sup>171-173</sup> There is no evidence in favor of prophylactic treatment of low-risk, asymptomatic patients, regardless of platelet count, but high-risk and symptomatic patients are treated to lower their platelet count to below 400,000/ $\mu$ L. In a cohort of 126 subjects with a median age of 31 years (range 5-40) smoking appeared to neutralize the protective effect of age less than 60 years.<sup>174</sup> The United Kingdom Medical Research Council Primary Thrombocythemia 1 Study has established hydroxyurea plus low-dose aspirin as the treatment of choice.<sup>175</sup> Whereas the actuarial rate of first thrombosis in an untreated high-risk population is approximately 26% at 2 years, the rate decreases to about 4% at 2 years with hydroxyurea and aspirin.<sup>175,176</sup> As the platelet count rises above 1.0 to 1.5 million/ $\mu$ L, there is increasing evidence of platelet dysfunction that may result in a risk of mucocutaneous hemorrhage in 35% or more of patients with essential thrombocythemia. This platelet count is an indication for cytoreductive therapy.<sup>164,170,177,178</sup> While several in-vitro platelet function abnormalities can be demonstrated,<sup>170</sup> the most significant functional consequence of hyperthrombocytosis is an acquired von Willebrand syndrome that results from the accelerated clearance of hemostatically competent large multimers of von Willebrand factor from the circulation.<sup>179-184</sup> Hemostatic function appears to improve with reduction of the platelet count.<sup>179,181,183,184</sup>

### Therapeutic Thrombocytapheresis in Primary Thrombocytosis

Rapid lowering of an elevated platelet count, using apheresis and/or chemotherapy, is indicated for patients with myeloproliferative disorders who present with clinical syndromes of microvascular thrombosis such as digital or cerebral ischemia.<sup>170</sup> Several case series and case reports have reported successful, rapid lowering of the platelet count in symptomatic patients in whom chemotherapy was either not an immediate option or was judged to have an insufficiently rapid effect.<sup>168,185-189</sup> Procedures in which 1.5 to 2.0 blood volumes are processed, and crystalloid replacement fluids are used to manage fluid balance, can lower the platelet count by 30% to 60%.<sup>6,185,189,190</sup> However, thrombocytapheresis without concomitant chemotherapy is not a practical means for controlling the platelet count beyond the acute setting.<sup>185,187,189</sup> Thus, symptomatic thrombocytosis is a Category II indication for thrombocytapheresis.<sup>1</sup> Weekly thrombocytapheresis, beginning in the 5th gestational week, has

been used in the management of a high-risk pregnant patient with essential thrombocythemia.<sup>190</sup>

### Therapeutic White Cell Apheresis

Hyperleukocytosis (white cell count  $>100,000/\mu$ L) with leukostasis is a Category I indication for therapeutic leukocytapheresis performed by centrifugation using discontinuous- or continuous-flow equipment.<sup>1,6,191-195</sup> Leukocytapheresis by selective adsorption techniques is currently under study in the United States and elsewhere for the treatment of inflammatory bowel disease, which ASFA has designated a Category P (pending) indication.<sup>1,6,196</sup>

### Leukocytapheresis for Hyperleukocytosis

Hyperleukocytosis is a risk factor for early mortality, often from pulmonary and/or central nervous system hemorrhage, in adults and children with acute myeloblastic leukemia.<sup>191,197</sup> It occurs in 5% to 13% of newly presenting cases of adult acute myelogenous leukemia and 12% to 25% of pediatric acute myelogenous leukemia.<sup>192,198</sup> The reported incidence in acute lymphoblastic leukemia ranges from 10% to 30%.<sup>198</sup> Mortality rates of 20% to 40% have been reported.<sup>198</sup> Children with acute lymphoblastic lymphoma are less susceptible to intracerebral hemorrhage if the white cell count is below 400,000/ $\mu$ L.<sup>191,199</sup> Clinical features of hyperleukocytosis with leukostasis include respiratory distress, hypoxemia, diffuse interstitial or alveolar infiltrates on chest x-ray, confusion, somnolence, stupor or coma, headache, dizziness, tinnitus, gait instability, or visual disturbances.<sup>198</sup> Physical examination may demonstrate papilledema, dilated retinal veins and/or retinal hemorrhages, cranial nerve defects, or meningeal signs.<sup>198</sup> Metabolic derangements caused by tumor lysis may include hyperkalemia, hyperuricemia, hypocalcemia, and hyperphosphatemia and may result in renal failure and early death.<sup>191,192,197</sup> Pulmonary, central nervous system, cardiac, and other organ morbidity results from obstruction of small vessels by aggregates of leukemic blasts with resulting infarction and hemorrhage.<sup>200</sup> Coagulopathy results from release of lysosomal enzymes from myeloid blasts, disseminated intravascular coagulation, and thrombocytopenia resulting from marrow failure.<sup>198-200</sup>

A standard treatment approach to hyperleukocytosis includes intravenous hydration, lowering of plasma uric acid using allopurinol or urate oxidase and, if urate oxidase is not used, administering intravenous sodium bicarbonate to alkalinize the urine.<sup>199</sup> Hydroxyurea may be prescribed to rapidly lower the total circulating nucleated cell count without precipitating a tumor lysis syndrome.<sup>198</sup> Induction chemotherapy may be used for this purpose but may precipitate tumor lysis syndrome and hemorrhage.<sup>200</sup> The processing of 1.5 to 2.0 blood volumes, using crystalloid or colloid fluids to maintain fluid balance, with or without a sedimenting agent such as 6% hydroxyethyl starch to enhance the separation of white cells from red cells, can reduce the circulating white cell count by up to 60%.<sup>6,193,194,201</sup> Leukocytapheresis before

initiation of definitive induction chemotherapy has been reported to lower the leukemic blast count to  $<100,000/\mu\text{L}$  in approximately 60% of patients, and to lower the incidence of early death (Day 21 after admission) by half, but does not affect long-term survival and may not prevent death from leukostasis in patients who present with severe symptoms of pulmonary or cerebral leukostasis before initiation of apheresis.<sup>191,193,195,201</sup> In any case, leukocytapheresis is not initiated without the above-mentioned measures (eg, hydration, uric acid lowering, and hydroxyurea) and should not be considered primary therapy in patients who present with high blast counts but without symptoms of leukostasis. Thus, leukostasis is appropriately classified as a Category II indication for leukocytapheresis.<sup>1,194,199,201</sup>

### Leukocytapheresis for Inflammatory Bowel Disease

Idiopathic inflammatory bowel disease principally refers to ulcerative colitis and Crohn's disease, similar but distinct disorders of intestinal inflammation.<sup>202-204</sup> Both may present with fever, diarrhea, rectal bleeding, weight loss, and abdominal pain.<sup>203</sup> Whereas perianal disease and anal strictures and fistulas are common in Crohn's disease, they are not features of ulcerative colitis. Also, ulcerative colitis is exclusive to the colon, while Crohn's disease involves the ileum in two-thirds of patients.<sup>203</sup> The majority of patients with ulcerative colitis exhibit positive serology for perinuclear-staining antineutrophil cytoplasmic antibodies but few are positive for anti-*Saccharomyces cerevisiae*.<sup>203-204</sup> Medical therapy for both disorders includes oral aminosalicylates, oral or parenteral steroids, immunomodulators (including azathioprine or mercaptopurine), and biological agents such as the anti-tumor necrosis factor agent infliximab.<sup>205-208</sup>

Interest in leukocytapheresis in the treatment of severe inflammatory bowel disease derives from the observed infiltration of the intestinal mucosa in these disorders by granulocytes and macrophages that produce marked mucosal tissue injury through the release of reactive oxide compounds, matrix metalloproteinases, and proinflammatory cytokines.<sup>203,208,209</sup> Two leukocyte adsorption columns are currently in use in Europe and Japan: a column of cylindrical nonwoven polyester fibers that preferentially binds granulocytes and lymphocytes (Celsorba, Asahi Kasei Kuraray, Tokyo, Japan) and a column of cellulose acetate beads that binds primarily granulocytes and monocytes (Adacolumn, JIMRO, Takasaki, Japan).<sup>196</sup> Reports of case series and small randomized (but not blinded) trials have claimed clinical benefit for both devices.<sup>1,196</sup> The Adacolumn is currently in Phase III pivotal trials in the United States; thus, inflammatory bowel disease is listed as a Category P (pending) indication for adsorption leukocytapheresis (designated "adoptive cytappheresis" by ASFA<sup>1,6</sup>) until the outcome of the trial is known.<sup>210</sup>

### Extracorporeal Photochemotherapy

Extracorporeal photochemotherapy (ECP, also referred to as "photopheresis") describes a procedure in which circulating

mononuclear cells are collected by centrifugal apheresis, exposed to 8-methoxypsoralen (8-MOP, a photoactivating agent), and then exposed to ultraviolet A (UVA) light. The treated cells are then reinfused into the patient. The only commercially available closed system is UVAR-XTS (Therakos, Exton, PA). A typical procedure, using single-needle access, consists of six cycles of processing 125 mL of blood or three cycles of processing 225 mL of blood to collect a buffy coat volume of approximately 240 mL. The instrument mixes the collected buffy coat cells with saline and 8-MOP (17  $\mu\text{L}$  per mL of buffy coat) and then pumps the cells through a 1-mm plastic film between two banks of fluorescent UVA lamps (352 nm, 1-2 J/cm<sup>2</sup>) to affect photoactivation. Whereas red cells and plasma are returned to the patient at the end of each collection cycle, the treated buffy coat product is returned following the last cycle. A full procedure is completed in approximately 3 hours.<sup>211,212</sup>

The precise mechanism of action of ECP is unknown,<sup>211,213</sup> but available evidence suggests that it works through a process of immunomodulation.<sup>212-214</sup> Photoactivated 8-MOP intercalates within nuclear DNA of normal and malignant T lymphocytes and causes apoptosis of treated T cells, but not treated monocytes, within 24 hours after ECP.<sup>215,216</sup> The two main immunologic consequences of phagocytosis of apoptotic cells appear to be induction of major histocompatibility Class I-restricted CD8+ cytotoxic T lymphocytes through the antigen-presenting activity of human dendritic cells and the elaboration by monocytes and macrophages of immunosuppressive cytokines including interleukin (IL)-10 and IL-1 receptor antagonist.<sup>213</sup> Further, ECP results in an increase in CD83+, CD86+ plasmacytoid (DC2) dendritic cells with a concordant diminution in CD80+, CD123+ monocytoïd (DC1) dendritic cells. The DC2 cells stimulate Th2 T-helper cells to secrete inhibitory cytokines (eg, IL-4, IL-10, IL-13) while inhibiting stimulation of Th1 T-helper cells that secrete proinflammatory cytokines (eg, IL-2, interferon- $\gamma$ ) and thus inhibiting Th1-mediated alloreactivity.<sup>214</sup> ECP also appears to result in an increase in a population of circulating CD4+, CD25+, CD69-CTLA-4+ regulatory T cells that are immunosuppressive and are involved in transplant tolerance.<sup>217</sup> Such induced immunologic responses to ECP are presumed to explain observed clinical benefit in diverse conditions such as cutaneous T-cell lymphoma, chronic graft-vs-host disease (GVHD), and cardiac transplant rejection.<sup>211-216,218</sup>

In 1998 the United States Centers for Medicare and Medicaid Services (CMS) approved Medicare reimbursement for ECP in the palliative treatment of skin manifestations of cutaneous T-cell lymphoma (CTCL) not responsive to other therapy. In 2006 CMS further determined that ECP is "reasonable and necessary" for treatment of acute cardiac allograft rejection and chronic GVHD refractory to standard immunosuppressive drug treatment.<sup>219</sup> ASFA has listed treatment of heart transplant rejection and cutaneous manifestations of GVHD as Category II indications for ECP, and erythrodermic CTCL and prophylaxis of heart transplant rejection as Category I indications for ECP.<sup>1,6</sup> Some

ECP regimens used in the treatment of the Category I and II indications<sup>1,6</sup> are described in Table 43-8.<sup>212,214,221-229</sup>

### Cutaneous T-Cell Lymphoma

ASFA has listed the treatment of erythrodermic CTCL as a Category I indication for ECP.<sup>1,6</sup>

#### Background

The CTCLs are a heterogeneous group of extranodal non-Hodgkin lymphomas of T-cell origin that target the skin.<sup>229,230</sup> Mycosis fungoides, the most common form of CTCL, accounts for almost half of all primary cutaneous lymphomas.<sup>230</sup> It is largely a disease of adults (median age 55-60 years at diagnosis) and typically presents as an indolent disorder that progresses slowly over years from patchy skin involvement to infiltrated plaques, tumors, and widespread disease.<sup>230</sup> Whereas localized (eg, nonerythrodermic) mycosis fungoides is adequately managed with topical therapies, the application of ECP in erythrodermic mycosis fungoides is recommended by ASFA and by the British Photodermatology Group and UK Skin Lymphoma Group.<sup>1,211</sup> Whereas limited-stage mycosis fungoides does not shorten life expectancy, advanced-stage disease may be associated with a 10-year disease-specific survival of 20%.<sup>230</sup> Sézary syndrome is defined as a triad of erythroderma, generalized lymphadenopathy, and the presence of neoplastic T cells (Sézary cells) in the

skin, lymph nodes, and peripheral blood.<sup>230</sup> Diagnostic criteria were recommended by the International Society for Cutaneous Lymphomas for the purpose of identifying patients with a worse prognosis compared to other erythrodermic forms of CTCL (notably erythrodermic mycosis fungoides).<sup>231</sup> These include  $\geq 1$  in 1000 circulating Sézary cells per  $\mu\text{L}$  of blood; a CD4/CD8 ratio  $\geq 10$  caused by expansion of circulating CD4+ T cells and/or aberrant loss or expression of pan-T-cell antigens (CD2, CD3, CD4, CD5) by flow cytometry; demonstration of a circulating T-cell clone by molecular or cytogenetic methods; or a cytogenetically abnormal T-cell clone.<sup>231</sup> The prognosis is poor, with median survival of 2 to 4 years and 5-year survival of approximately 24%.<sup>229</sup> A retrospective cohort study from Stanford of 106 patients with erythrodermic mycosis fungoides and Sézary syndrome identified age  $\geq 65$  years, clinical Stage IV and circulating Sézary cells  $\geq 5\%$  of total lymphocytes as independent negative prognostic factors for survival.<sup>232</sup> Median survivals of patients with none, one, or more than one of these adverse prognostic factors were 122 months ( $n = 36$ ), 44 months ( $n = 39$ ), and 18 months ( $n = 31$ ), respectively ( $p < 0.005$ ).<sup>232</sup>

#### Extracorporeal Photochemotherapy in the Treatment of Cutaneous T-Cell Lymphoma

The 1987 report by Edelson et al<sup>233</sup> of a successful pilot trial of ECP, in which 27 of 37 treatment-resistant patients with CTCL experienced an average 64% decrease in cutaneous involvement after  $22 \pm 10$  weeks, led to FDA approval of ECP for the treatment of advanced CTCL later that year.<sup>234</sup> Long-term follow-up of the original 29 patients with erythrodermic CTCL from the pilot study of Edelson et al<sup>233</sup> reported that median survival of the treated patients was 60.33 months from diagnosis and 47.9 months from the start of ECP. Four of the six patients who achieved complete responses in the original study remained in complete remission.<sup>235</sup> A retrospective study from London compared survival of patients with Sézary syndrome who had not received ECP to survival of patients who had.<sup>236</sup> Median survival was 39 months (range 1-138) in the ECP group ( $n = 29$ ) and 26.5 months (12-67) in the non-ECP group ( $n = 15$ ), a nonsignificant difference ( $p = 0.12$ ).<sup>236</sup> All patients had been demonstrated to have a circulating T-cell clone by testing based on polymerase chain reaction, whereas this was not the case in Edelson's study.<sup>233</sup>

Between 1987 and 2001, 21 studies were reported in which a total of 485 patients were treated using ECP.<sup>211,212,237</sup> Although most patients in these studies had erythrodermic CTCL, most studies did not report the response rates separately for the erythrodermic subjects.<sup>237</sup> In addition, responses were defined as  $>25\%$  skin clearing in some studies and  $>50\%$  skin clearing in others, and complete responses were defined as either 75% to 100% skin clearing,  $>90\%$  skin clearing, or 100% skin clearing.<sup>212</sup> Nonetheless, overall response rates reported in these studies ranged from 31% to 87.5%, and complete response rates ranged from 0% to 54% (20%-30% in most studies).<sup>211,212,237</sup> The variability in response rates has been attributed to

**Table 43-8.** Regimens of Extracorporeal Photochemotherapy (ECP)

| Indication                    | Treatment                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
|-------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Cutaneous T-cell lymphoma     | ECP on 2 consecutive days once per month; continue for 6 months before declaring treatment failure <sup>212,229</sup>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| Cardiac allograft rejection   | Prophylaxis <sup>220,221</sup> <ul style="list-style-type: none"> <li>Month 1 after transplant: ECP day 1 and 2, 5 and 6, 10 and 11, 17 and 18, 27 and 28</li> <li>Month 2 and 3 after transplant: ECP on 2 successive days every 2 weeks</li> <li>Month 4-6 after transplant: ECP on 2 successive days every 4 weeks</li> </ul> <p>Acute (moderate) rejection<sup>222,223</sup></p> <ul style="list-style-type: none"> <li>ECP procedures on 2 consecutive days at weekly episode intervals as needed to resolve rejection as indicated by endomyocardial biopsy</li> </ul> <p>Recurrent/refractory rejection<sup>224,225</sup></p> <ul style="list-style-type: none"> <li>ECP on 2 consecutive days weekly for 1 month, then every 2 weeks for 2 months, then monthly for 3 months (total of 22 procedures)</li> </ul> <p>Chronic rejection<sup>226</sup></p> <ul style="list-style-type: none"> <li>Beginning within 1 month of transplantation: ECP on 2 successive days every 4 weeks for 12 months, every 6 weeks for next 6 months, every 8 weeks during next 6 months</li> </ul> |
| Chronic graft-vs-host disease | ECP on 2 consecutive days every 1-2 weeks, then consider monthly interval; treat at least 6 months before declaring treatment failure <sup>214,227,228</sup>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |

differences in entry criteria, prior or concurrent therapy, duration between diagnosis and application of ECP, the ECP protocol followed, and definition of response.<sup>211</sup> In one analysis of 23 patients with Sézary syndrome treated with ECP as the sole therapy for up to 1 year,<sup>237</sup> improvement in skin score was positively associated with a reduction in circulating Sézary cells as a percentage of total white cell count ( $p = 0.03$ ). Neither the absolute change in total white cell count, lymphocyte count, CD34 count, CD8 count, nor the Sézary count distinguished the 13 patients who responded to treatment from the nonresponders. Nor were age, gender or prior treatment with chemotherapy associated with responder status. Baseline lymphocyte count was associated with change in skin score at 6 months ( $p = 0.046$ ) but not at 12 months. Sézary cell count as a percentage of total white cell count was the only baseline variable that was a significant predictor of responder status at 6 months: a 1% increase in % Sézary cells at baseline was associated with an odds ratio of 1.07 of becoming a responder ( $p = 0.021$ ) at 6 months but not at 12 months.<sup>238</sup>

Cardiac Allograft Rejection

ASFA has listed prophylaxis of heart transplant rejection as a Category I indication for ECP and treatment of heart transplant rejection as a Category II indication for ECP.<sup>1,6</sup> The Category I designation for heart transplant rejection prophylaxis is apparently determined on the basis of two small randomized pilot studies.<sup>220,221</sup>

Background

Between 1990 and 2006, approximately 3000 to 4500 heart transplant procedures were reported each year to the registry of the International Society for Heart and Lung Transplantation.<sup>239</sup> Between 1 January 2004 and 30 June 2006, 41% of heart transplant procedures were performed for heart failure caused by

coronary disease and 45% for noncoronary cardiomyopathy, with an average recipient age of (mean  $\pm$  SD)  $50.7 \pm 12.5$  years (range 18–77).<sup>239</sup> Treatment for an episode of allograft rejection was associated with a 26% increase in mortality risk before hospital discharge and a 48% increase in mortality risk after discharge but within the first postoperative year.<sup>239</sup> From January 1992 to June 2006, acute allograft rejection accounted for 12% of deaths, and after 5 years allograft vasculopathy and late graft failure (presumed to be the result of chronic rejection and consequent allograft vasculopathy) combined to account for 30% of deaths.<sup>239</sup> Immunosuppressive therapy to prevent allograft rejection has included perioperative lymphocyte antibody (polyclonal antilymphocyte or antithymocyte globulin, OKT3, or IL-2 receptor antibodies), and chronic postoperative calcineurin inhibitors (eg, tacrolimus, cyclosporine), mycophenolate mofetil, sirolimus, and others.<sup>239</sup>

Cardiac allograft rejection is defined and classified on the basis of histologic findings obtained through surveillance endomyocardial biopsy following transplantation, and may not present as symptoms of allograft dysfunction.<sup>240</sup> Because of the association between rejection, graft failure, and death, biopsy-detected evidence of rejection should result in adjustment in the immunosuppressive regimen in order to successfully resolve the episode of rejection.<sup>222,225,239,240</sup> Thus CMS has only approved reimbursement for severe, drug-refractory rejection.<sup>219</sup> The histologic criteria for acute cellular rejection are summarized in Table 43-9.

Extracorporeal Photochemotherapy in the Treatment of Cardiac Allograft Rejection

Studies in support of ECP for treatment of cardiac allograft rejection focus on the effects of ECP on endomyocardial biopsy findings, rather than on survival or graft function.<sup>211,219</sup> Two pilot studies have shown evidence that the risk of acute cellular

Table 43-9. Initial and Modified International Society for Heart Transplantation Criteria for Cellular Allograft Rejection

| Severity           |         | 1990 <sup>241</sup>                                                                                                        | 2005 <sup>242</sup> |                                                                                                 |
|--------------------|---------|----------------------------------------------------------------------------------------------------------------------------|---------------------|-------------------------------------------------------------------------------------------------|
| No rejection       | Grade 0 | No rejection                                                                                                               | Grade 0 R           | No rejection                                                                                    |
| Mild rejection     | Grade 1 | A – Focal:<br>Focal perivascular and/or interstitial infiltrate without myocyte damage                                     | Grade 1 R           | Interstitial and/or <i>perivascular</i> infiltrate up to one focus of myocyte damage            |
|                    |         | B – Diffuse:<br>Diffuse infiltrate without myocyte damage                                                                  |                     |                                                                                                 |
| Moderate rejection | Grade 2 | Focal:<br>One focus of infiltrate with associated myocyte damage                                                           | Grade 2 R           | Two or more foci of infiltrate with associated myocyte damage                                   |
|                    | Grade 3 | A – Focal:<br>Multifocal infiltrate with myocyte damage<br>B – Diffuse:<br>Diffuse infiltrate with myocyte damage          |                     |                                                                                                 |
| Severe rejection   | Grade 4 | Diffuse, polymorphous infiltrate with extensive myocyte damage with vasculitis and with or without edema and/or hemorrhage | Grade 3 R           | Diffuse infiltrate with multifocal myocyte damage with or without edema, hemorrhage, vasculitis |

rejection episodes can be decreased by incorporating ECP into the prophylactic immunosuppressive regimen of cardiac allograft recipients without increasing the risk of infection caused by immunosuppression.<sup>220,221</sup> Another prospective pilot trial has reported that development of panel-reactive antibodies (responsible for chronic rejection) and coronary artery intimal hyperplasia (a pathogenetic mechanism of graft failure in chronic rejection) are mitigated by the addition of ECP to the post-transplant immunosuppressive regimen for the first 2 years after transplant surgery.<sup>226</sup>

Dall'Amico and colleagues in Padua, Italy reported a prospective pilot trial in eight patients, with recurrent acute rejection episodes despite immunosuppression, who were treated with 2 consecutive days of ECP every 4 weeks for 6 months.<sup>243</sup> Seven benefited with a reduction in the number and severity of rejection episodes; reduction in daily prednisone, cyclosporine, or azathioprine doses; and improvement on endomyocardial biopsy specimens. The investigators conducted two follow-up studies—one a prospective uncontrolled study in 22 patients with two or more acute rejection episodes<sup>224</sup> and the other a prospective uncontrolled study of 11 patients with recurrent severe acute rejection episodes.<sup>244</sup> They reported that an intensive regimen of ECP (see Table 43-9) is highly effective in resolving and preventing recurrent acute rejection refractory to conventional immunosuppression, but cautioned that most acute rejection episodes, particularly nonsevere episodes, could be effectively managed using medical regimens without ECP.<sup>219</sup> Other small case series and case reports have presented corroborating data.<sup>222,223,225,245,246</sup> Evidence to support ECP in the management of renal or lung transplant rejection are insufficient to recommend its use in these settings.<sup>1,211</sup>

### Graft-vs-Host Disease

The ASFA 2007 evidence-based review of apheresis therapies lists skin manifestations of GVHD as a Category II indication for ECP.<sup>1,6</sup> CMS considers ECP reasonable and necessary for the treatment of chronic GVHD that is refractory to standard immunosuppressive drug therapy.<sup>219</sup>

### Background

GVHD in hematopoietic progenitor cell transplant recipients occurs when T cells of donor origin (either transplanted with, or that develop from, the graft) interact with tissue in the HLA-matched but genetically nonidentical host.<sup>247</sup> Classical acute GVHD develops within 100 days of transplantation with skin manifestations that vary from an erythematous morbilliform rash to epidermal necrolysis, mucosal inflammation causing diarrhea and abdominal cramping, and abnormalities of liver function tests.<sup>247,248</sup> GVHD that develops beyond 100 days of transplantation, or persists more than 3 months, is traditionally referred to as chronic GVHD, and is characterized by an oral, ocular, and mucous membrane sicca syndrome; skin involvement; scleroderma; bronchiolitis obliterans; joint contractures; myofasciitis; esophageal stricture; or other fibrotic

complications in various organ systems.<sup>247,249</sup> The cumulative incidence of acute GVHD is approximately 12% to 75%, and the cumulative incidence of chronic GVHD is approximately 15% to 70% after hematopoietic cell transplantation, depending on whether the donor-recipient pair are related or unrelated and on whether a myeloablative or nonmyeloablative conditioning regimen was used.<sup>249-251</sup>

The onset of acute GVHD may be delayed beyond 100 days in some patients such as those who receive nonmyeloablative conditioning regimens, and chronic GVHD may present within the first 2 months after transplantation in certain circumstances.<sup>250,251</sup> A consensus conference of the National Institutes of Health has proposed a categorization of GVHD based on these observations, taking into account the clinical overlap syndromes between the two (see Table 43-10).<sup>252</sup> Accurate diagnosis and staging is important in that recurrent or late onset acute GVHD may not require prolonged therapy as is required with chronic GVHD, while overlap syndromes may require shorter courses of typical treatments for chronic GVHD.<sup>253</sup>

### Extracorporeal Photochemotherapy in the Treatment of GVHD

The application of ECP to the treatment of GVHD has been extensively reviewed.<sup>211,219,254-256</sup> There are no controlled, blinded, randomized trials, and most reports have been case reports, case series, or retrospective analyses, although some prospective analyses are available.<sup>219,254</sup> Among the larger prospective reports is one from a London group that treated 28 patients who had developed steroid-refractory chronic GVHD following HLA-matched allogeneic marrow or peripheral blood progenitor cell transplant.<sup>227</sup> Among the patients, 27 were classified as having extensive chronic GVHD<sup>257</sup> and 20 had involvement of more than 50% of their skin surface. Patients were given ECP on 2 consecutive days every 2 weeks for the first 4 months, and monthly thereafter. ECP was initiated a median of 34 months (range 10-167) after transplantation and 23 months (range 2-164) from the onset of chronic GVHD. Of the 21 patients with cutaneous involvement who were evaluable, a 25% reduction in skin involvement was noted in eight (38%) after 3 months and in 10 (48%) after 6 months, and a statistically nonsignificant improvement in liver function tests was noted.<sup>227</sup> The authors contrasted their results to those of a retrospective report of 15 patients with extensive chronic GVHD who received ECP on a similar schedule, beginning a median of 12 months (range 3-44) after transplantation. Among those 15 patients, 12 (80%) achieved complete resolution of cutaneous manifestations of GVHD, seven of 10 (70%) patients with liver involvement had complete responses, and all 11 patients with oral mucosal disease had complete responses.<sup>258</sup>

A group from Boston enrolled 25 patients with extensive, steroid-refractory chronic GVHD in a prospective uncontrolled trial to evaluate ECP for skin and visceral involvement.<sup>259</sup> ECP was performed in the outpatient setting for 2 consecutive days every 2 weeks on 17 patients and weekly on eight patients who

lived a long distance from the treatment site. The median time from transplantation to initiation of ECP was 790 days (range 242-2928), and ECP continued, until best response or disease progression, for a median of 9 months (range 3-24). Sixteen patients (64%) responded ( $\geq 50\%$  improvement in skin rash;  $\geq 50\%$  improvement in bilirubin) in at least one site of disease. Twenty improved with respect to cutaneous involvement, including softening of sclerodermatous changes in six. Six of 13 with oral mucosal ulcers experienced healing of their ulcers during

ECP. Three of six patients with joint involvement had increased range of motion.<sup>259</sup>

An Italian group evaluated ECP in a prospective, uncontrolled study of 32 patients with chronic GVHD who began treatment a median of 5 months (range 1-56) after hematopoietic cell transplantation.<sup>260</sup> All but five had cutaneous involvement. The treatment schedule was the same as that used by the London group.<sup>227</sup> The investigators reported that 16 patients (50%) achieved a complete response and were able to have their

**Table 43-10.** Syndromes of Acute and Chronic Graft-vs-Host Disease (GVHD)<sup>252</sup>

|                                   | Clinical Onset<br>After Transplantation | Features of Acute GVHD                                                                                                                                                                                                                                                                                                                                                                                     | Features of<br>Chronic GVHD*                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |
|-----------------------------------|-----------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Classical acute GVHD              | $\leq 100$ days                         | Skin                                                                                                                                                                                                                                                                                                                                                                                                       | None                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
| Persistent, recurrent, late onset | $> 100$ days                            | <ul style="list-style-type: none"> <li>• Erythema</li> <li>• Maculopapular rash</li> </ul> Mouth <ul style="list-style-type: none"> <li>• Gingivitis</li> <li>• Mucositis</li> <li>• Erythema</li> <li>• Pain</li> </ul> GI tract <ul style="list-style-type: none"> <li>• Anorexia</li> <li>• Nausea</li> <li>• Vomiting</li> <li>• Diarrhea</li> <li>• Ileus</li> <li>• Cholestatic hepatitis</li> </ul> |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
| Classical chronic GVHD            | No time limit                           | None                                                                                                                                                                                                                                                                                                                                                                                                       | Skin <ul style="list-style-type: none"> <li>• Poikiloderma</li> <li>• Lichen planus</li> <li>• Scleroderma</li> </ul> Mouth <ul style="list-style-type: none"> <li>• Lichen-type features</li> <li>• Hyperkeratotic plaques</li> <li>• Restricted opening</li> </ul> Genitalia <ul style="list-style-type: none"> <li>• Lichen-planus</li> <li>• Vaginal stenosis</li> </ul> GI tract <ul style="list-style-type: none"> <li>• Esophageal web</li> <li>• Esophageal strictures</li> </ul> Lung <ul style="list-style-type: none"> <li>• BOOP (biopsy)</li> </ul> Muscle <ul style="list-style-type: none"> <li>• Fasciitis</li> </ul> Skeletal <ul style="list-style-type: none"> <li>• Joint stiffness</li> <li>• Contractures</li> </ul> |
| Overlap syndrome                  | No time limit                           | Yes; same as above                                                                                                                                                                                                                                                                                                                                                                                         | Yes; same as above                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |

\*Any of these features are considered sufficient to establish a diagnosis of chronic GVHD

GI = gastrointestinal; BOOP = bronchitis obliterans with organizing pneumonia.

immunosuppressive drug doses decreased by at least half (the “determinant” group). In seven patients (22%) ECP was declared ineffective in that either progression was observed in at least one involved organ system, it was necessary to increase the dose of immunosuppressants, or immunosuppressants were not reduced by >50% but no clinical complete responses were obtained. The remaining nine patients (28%) were considered responders if their outcomes were better than the “ineffective” group but not as good as the “determinant” group. Thus, with an overall response rate of 78%, the authors concluded that ECP is beneficial for steroid-refractory chronic GVHD.<sup>260</sup>

A pilot study from Vienna is the principal source of data regarding the use of ECP to treat steroid-refractory acute GVHD.<sup>261</sup> In this prospective, unblinded, nonrandomized study, 21 patients who underwent marrow (n = 19) or peripheral blood progenitor cell (n = 2) transplantation and who experienced acute GVHD unresponsive to corticosteroids and cyclosporin A, were treated with ECP on 2 consecutive days (one cycle) at 1- to 2-week intervals until improvement and then every 2 to 4 weeks until maximal response was reached. ECP was tapered thereafter on an individual basis. The onset of acute GVHD occurred a median of 19 days (range 13-33) after transplantation and drug therapy was given for a median of 21 days (range 9-49) before the start of ECP. The maximal response to ECP was achieved after a median of four cycles (range 1-13) or 2 months (range 0.5-6) of treatment. Complete resolution of acute GVHD was achieved in 60% of patients after 3 months of ECP. Three months after initiation of ECP, complete responses were obtained in 60% of patients with cutaneous manifestations, 67% of patients with liver manifestations, and no patients with gut manifestations of acute GVHD.<sup>261</sup> Overall transplant-related mortality was similar to that achieved using conventional second-line immunosuppressive therapy for steroid-resistant acute GVHD.<sup>253</sup>

Corroborating data in support of the benefit of ECP in mucocutaneous chronic GVHD are provided by smaller prospective and several retrospective studies.<sup>262-267</sup> At present, the report of the combined workshop of the British Photodermatology Group and the UK Skin Lymphoma Group on evidence-based practice of photopheresis considers the available evidence sufficient to recommend ECP for the treatment of steroid-refractory chronic GVHD but not for acute GVHD.<sup>211</sup> CMS also supports the use of ECP as reasonable and necessary for patients with chronic GVHD whose disease is refractory to standard immunosuppressive drug treatment but does not consider acute GVHD in its analysis.<sup>219</sup> ASFA considers skin manifestations to be a Category II indication for ECP but does not distinguish chronic from acute in its recommendation.<sup>1,6</sup>

## Specialized Therapeutic Plasma Processing

In its 2007 evidence-based review of apheresis therapies, ASFA lists 30 Category I, II, or P indications for therapeutic plasma processing, 24 of which are indications for therapeutic plasma exchange (TPE).<sup>1</sup> The physiology of TPE and its indications are

addressed in Chapter 42. The discussion herein focuses on therapeutic procedures that, rather than removing whole plasma, are designed to remove a specified fraction of the plasma and return the processed plasma to the patient. A pathogenic plasma substance (for example, a specific autoantibody) is usually present at relatively low levels in the circulation: a typical plasma exchange may remove 150 g of healthy plasma protein in order to eliminate 1 to 2 g of offending substance.<sup>268</sup> To minimize the sacrifice of healthy plasma proteins, selective extraction of pathologic plasma constituents has been proposed for the treatment of patients with disorders that might otherwise be treated using TPE.<sup>269</sup>

### Immunoadsorption Apheresis

Immunoadsorption systems employ the principle of affinity chromatography and make use of immobilized sorbents or ligands that have enhanced or specific binding affinity for a specific antigen, antibody, immune complex, or other substance in the patient's circulation.<sup>269,270</sup> Examples include 1) staphylococcal protein A or sheep antihuman immunoglobulin (IgG) antibody for extraction of IgG and immune complexes from the circulation, 2) sheep antihuman low-density lipoprotein (LDL) or apolipoprotein B antibody for extraction of LDL, 3) synthetic blood group substances for removal of ABO isoagglutinins, and 4) DNA for removal of DNA antibody. Two immunoadsorption systems that have received approval from the US FDA are the staphylococcal protein A-agarose column (Immunosorba, Fresenius HemoCare, Redmond, WA),<sup>271</sup> and the staphylococcal protein A-silica column (Prosorba, Fresenius HemoCare).<sup>272</sup>

Protein A is a cell-wall constituent of the Cowan I strain of *Staphylococcus aureus*. Mammalian IgG binds to five homologous regions at its amino terminus, but interaction of protein A with other plasma proteins is insignificant. Protein A interacts strongly with IgG1, IgG2, and IgG4, but only to a variable extent with IgG3, IgM, and IgA. The affinity of protein A is higher for complexed IgG than for free IgG. Processing of 2.5 plasma volumes using a protein A-agarose column resulted in a 97% reduction in IgG1, a 98% reduction in IgG2, a 40% reduction in IgG3, a 77% reduction in IgG4, a 56% reduction in IgM, and a 55% reduction in IgA, while plasma levels of albumin, fibrinogen, and antithrombin were reduced by less than 20%.<sup>270</sup> Thus, in principle, plasma adsorption with protein A affinity columns permits the processing of more plasma than does TPE without unacceptable loss of other essential plasma constituents.<sup>268</sup>

The commercial distribution of both protein A immunoadsorption systems in the United States was discontinued in 2006.<sup>6</sup> The use of these devices in a variety of clinical conditions has been extensively reviewed.<sup>268</sup> A brief discussion of current Category II or P indications<sup>1,6</sup> for therapeutic immunoadsorption follows.

### Immune Thrombocytopenic Purpura

Immune thrombocytopenic purpura (ITP, also known as idiopathic thrombocytopenic purpura) presents with thrombocytopenia and mucocutaneous bleeding and is caused by accelerated



clearance of IgG-coated platelets that interact with Fc $\gamma$  receptors on tissue macrophages, primarily in the spleen and liver.<sup>273</sup> Antibodies against platelet surface glycoproteins, typically glycoprotein IIb/IIIa or Ib/IX, may be naturally occurring, a result of ingestion of medication, or a manifestation of autoimmune or lymphoproliferative disorders.<sup>273</sup> Treatment focuses on immunosuppressants, with corticosteroids serving as traditional first-line therapy, and splenectomy often used in the case of steroid failure.<sup>273,274</sup> Other effective or promising treatments, including intravenous IgG, anti-D immunoglobulin, and rituximab have proven more popular among practitioners than immunoadsorption for the treatment of otherwise refractory cases,<sup>273-276</sup> and a new generation of therapies for ITP may yet supercede current treatment approaches.<sup>277</sup> Immunoadsorption with the Prosorba column was reported effective in a group of 72 patients with ITP who had failed to improve on at least two other therapies.<sup>276</sup> An increased platelet count was achieved in 33 (46%) of refractory ITP patients who had failed at least two other treatments (including steroids and splenectomy), but responses in seven of the 33 responders lasted less than a month.<sup>276</sup> The remaining (79%) responding patients did not experience a relapse of thrombocytopenia over a mean follow-up period of 8 months.<sup>276</sup> In another report, six of 10 platelet-refractory, alloimmunized patients achieved at least a doubling of their platelet count after Prosorba treatment.<sup>278</sup> In both reports, many of the patients continued to receive other therapies, and there was no control group. Furthermore, little or no corroborating data have been published. Nonetheless, ASFA considers refractory ITP a Category II indication for immunoadsorption.<sup>1,6</sup>

### Rheumatoid Arthritis

The Prosorba column was approved by the FDA "... for use in the therapeutic reduction of the signs and symptoms of moderate to severe rheumatoid arthritis (ra) in adult patients with long-standing disease who have failed or are intolerant to disease-modifying anti-rheumatic drugs (dmards)."<sup>279</sup> The serendipitous observation of apparent improvement in symptoms of patients with rheumatoid arthritis who were receiving Prosorba treatment for ITP resulted in a pilot study in which nine of 11 patients with refractory rheumatoid arthritis showed apparent benefit.<sup>280</sup> A properly conducted pivotal randomized trial assessed Prosorba vs placebo in 91 patients who had failed to respond to methotrexate or at least two other second-line drugs.<sup>281</sup> Efficacy was assessed 7 to 8 weeks after completion of 12 weekly true or sham treatments. An independent data safety monitoring board stopped the trial after a planned interim analysis because 31.9% of the 47 patients in the Prosorba arm reached the defined therapeutic endpoint while only 11.4% of the 44 sham-treated patients did ( $p = 0.019$ ). Subsequent reported experience was consistent with the study findings.<sup>282</sup> As it happened, Prosorba was introduced almost simultaneously with a new generation of anti-rheumatic drugs (including leflunomide, entercept, and infliximab) and these quickly overshadowed Prosorba in the management of severe rheumatoid arthritis.<sup>283,284</sup> Although it has been suggested

that Prosorba may be preferable for patients in whom chronic infection renders potent immunosuppressive agents less desirable,<sup>285</sup> its market withdrawal renders the issue moot, at least in the United States.

### Coagulation Factor Inhibitors

Alloantibody inhibitors to coagulation factors may arise in approximately 20% of patients with severe hemophilia A (Factor VIII deficiency) and 12% of patients with severe hemophilia B (Factor IX deficiency).<sup>286</sup> The risk may be higher in patients treated solely with recombinant, as opposed to plasma-derived, clotting factor replacement.<sup>287</sup> Autoantibody inhibitors, most typically directed against Factor VIII ("acquired hemophilia") occur at a rate of approximately one person per million per year, and 40% to 50% of cases are seen in association with other conditions including pregnancy, autoimmune disorders, hematologic or other malignancies, and use of drugs such as antibiotics or anticonvulsants.<sup>288</sup>

Nilsson<sup>289</sup> first reported the use of protein A-Sepharose (Sigma-Aldrich Co., St. Louis, MO) adsorption, in combination with immunosuppression, in the management of a patient with hemophilia B and a high-titer Factor IX inhibitor. A number of case reports and small case series have since reported successful lowering of inhibitor titer using the Prosorba, Immunosorba, or TheraSorb-Ig [sheep antihuman immunoglobulin coupled to Sepharose (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany)] columns in patients with congenital and acquired hemophilia.<sup>290-294</sup> In these procedures, approximately 2.5 to 3.0 plasma volumes are processed at up to 50 mL/minute (plasma flow rate), and a series of one to six procedures is performed at 1- to 2-day intervals, thus lowering inhibitor titers by as much as 90% to 100%.<sup>290,294</sup> Patients with high-titer inhibitors must receive concurrent immunosuppressive treatment in order to prevent rebound of the inhibitor after several days or in order to induce tolerance to Factor VIII or IX.<sup>288,290</sup>

Because of the use of concurrent medication (eg, Factor VIIa, activated prothrombin complex concentrates, large doses of human Factor VIII concentrates, porcine Factor VIII) it is difficult to assess the specific contribution of the apheresis procedure to the achievement of hemostatic control in reported cases.<sup>288</sup> Because of this, and in the absence of clinical trial data, ASFA has designated management of coagulation factor inhibitors a Category III indication for immunoadsorption apheresis (paucity of data).<sup>1,6</sup> Immunoadsorption may be useful as an adjunct to early management of patients with inhibitors who are hemorrhaging or who need surgery too emergently to await the outcome of immunosuppressive therapy and who do not respond adequately to factor replacement therapy.<sup>290,292-294</sup> And as stated earlier, the removal of the relevant devices from the US market renders the issue moot in the United States.

### Dilated Cardiomyopathy

Dilated cardiomyopathy (DCM) describes a cardiac disorder in which the left ventricle is dilated and exhibits impaired contraction.

It may be idiopathic, familial, post-viral, alcohol- or drug-induced, or related to other cardiac disease. Both ventricles may be affected. It presents with heart failure, often progressive, and may be complicated by arrhythmias, thromboembolic events and sudden death.<sup>295</sup> It is the most frequent antecedent cause of heart transplantation throughout the world.<sup>239</sup> Approximately 60% to 80% of patients with DCM harbor autoantibodies directed against cardiac myosin heavy chain, myocardial  $\beta_1$ -adrenergic receptors, or other cardiac tissue with a predominance of antibodies of the IgG 3 subclass.<sup>296-301</sup> Antibodies directed against cardiac tissue targets, similar to those involved in DCM, induce structural and functional changes consistent with DCM in animal models.<sup>302,303</sup>

The potential relevance of heart antibodies to clinical DCM was explored in a pilot study of eight patients with DCM and advanced congestive heart failure who were subjected to a series of four or five immunoadsorption procedures per week over 2 weeks using the Therasorb-Ig system.<sup>304</sup>  $\beta_1$ -adrenoreceptor autoantibody levels decreased and heart failure symptoms improved in seven of the subjects, but the effect was transient: autoantibodies and symptoms were restored to baseline 75 days after completion of immunoadsorption.<sup>304</sup> In a companion pilot trial, nine patients were subjected to daily immunoadsorption treatments for 5 days using Therasorb-Ig.<sup>305</sup> Patients received 35 g of intravenous polyclonal immunoglobulin after the final procedure. Once again, plasma levels of  $\beta_1$ -adrenoreceptor autoantibody were substantially reduced and significant improvement in hemodynamic parameters (including cardiac output, mean arterial pressure, mean pulmonary arterial pressure, left ventricular filling pressure, and systemic vascular resistance) was demonstrated, thus providing objective preliminary evidence of the clinical benefit of antibody removal by immunoadsorption.<sup>305</sup> Immunoadsorption was also shown to diminish histologic evidence of myocardial inflammation in another cohort of 25 patients with DCM.<sup>306</sup>

In a randomized, prospective but nonblinded trial, 18 patients with DCM and advanced heart failure were randomly assigned to either best medical therapy (control group) or best medical therapy with the addition of immunoadsorption.<sup>307</sup> During the first course of immunoadsorption, procedures were performed on 3 consecutive days and patients received 0.5 g/kg of polyclonal IgG by intravenous infusion. Three subsequent courses of immunoadsorption, performed on 2 consecutive days at 4-week intervals were also followed by infusions of intravenous IgG at the same dose. Immunoadsorption was performed using the Therasorb-Ig column. After the first course of immunoadsorption/IgG, left ventricular ejection fraction, cardiac index, stroke volume index, and systemic vascular resistance improved significantly in the treatment group and these changes remained evident after the final series. Improvement in symptoms and functional status paralleled the hemodynamic changes. The control group demonstrated no hemodynamic improvement at the end of the 3-month study.  $\beta$ -adrenergic receptor antibodies decreased by >80% after the first course of immunoadsorption but tended to rise between monthly courses of treatment.

Although the studies described above demonstrated a response to immunoadsorption in patients with DCM and severe heart failure, subjects in those studies received a cumulative dose of intravenous IgG equal to that used in another study in which patients with DCM and advanced heart failure received a single course of 2 g/kg of intravenous IgG and showed persistent hemodynamic and functional improvement for a year of follow-up.<sup>308</sup> Two randomized, blinded, placebo-controlled trials yielded conflicting results regarding IgG infusion in DCM. One trial, in which patients initially received a total of 2 g/kg followed by 0.4 g/kg monthly for 5 months, demonstrated significant improvement in left ventricular ejection fraction in the treatment group but not the placebo group.<sup>309</sup> A second trial, in which patients received a single course totaling 2 g/kg demonstrated no effect of intravenous IgG on left ventricular ejection fraction after 6 or 12 months (although both the treatment and the placebo group showed improvement).<sup>310</sup> In a pilot study of four patients with DCM who received immunoadsorption with Immunosorba columns, procedures were performed daily for 5 consecutive days and no intravenous IgG was given.<sup>311</sup> Total IgG decreased by 95% and IgG3 decreased by 61%. Functional parameters including mean left ventricular ejection fraction, left ventricular end-systolic and diastolic volumes, and global end-systolic strain improved but not to a statistically significant degree. However, a quality of life assessment demonstrated significant improvement at 6 months.<sup>311</sup> ASFA has designated DCM a Category P (pending) indication for immunoadsorption apheresis.<sup>1,6</sup>

## Selective Extraction of Low-Density Lipoproteins

### Background

Familial hypercholesterolemia (FH), a dominantly inherited disorder, is a major cause of death or early disability resulting from premature atherosclerotic heart and peripheral vascular disease.<sup>312,313</sup> Autosomal dominant FH is caused by mutations in the gene for the LDL receptor, with frequencies of 1:500 for heterozygotes and 1:1,000,000 for homozygotes.<sup>313</sup> Clinical features including xanthomas, xanthelasmas, corneal arcus, and the occurrence of coronary heart disease, stroke, and death are common in the fourth or fifth decade of life.<sup>312,313</sup> A serum LDL-cholesterol level below 100 mg/dL (achieved through diet, lifestyle modification, and 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibiting drugs or statins) can lower cardiovascular morbidity and mortality in high-risk patients, but a sizable minority of high-risk patients fail to meet LDL-cholesterol-lowering goals by this approach.<sup>314,315</sup>

Manual, then automated, plasmapheresis was successfully employed as an adjunct to lipid-lowering therapy beginning over 40 years ago. However, the kinetics of restoration of plasma lipid levels and the unwanted lowering of essential plasma proteins (eg, fibrinogen, albumin) rendered this approach somewhat impractical for long-term therapy.<sup>312,316</sup> The development of devices that permit a more selective removal of plasma LDL-cholesterol and related substances has provided a practical apheresis-based approach to managing statin-resistant patients

in conjunction with statins and other medical therapies.<sup>316</sup> Two apheresis systems for selective removal of LDL-cholesterol are FDA-approved for use in the United States.<sup>317,318</sup>

ASFA has designated FH a Category I indication for LDL apheresis in homozygotes and a Category II indication for LDL

**Table 43-11.** FDA-Approved Indications for Use of LDL Apheresis to Acutely Lower Plasma LDL-Cholesterol Levels in Certain High-Risk Patient Populations<sup>317,318</sup>

| Patient Group | Description*                                                                                                                         |
|---------------|--------------------------------------------------------------------------------------------------------------------------------------|
| Group A       | Functional hypercholesterolemic homozygotes with plasma level of LDL-cholesterol >500 mg/dL                                          |
| Group B       | Functional hypercholesterolemic heterozygotes with plasma level of LDL-cholesterol ≥300 mg/dL                                        |
| Group C       | Functional hypercholesterolemic heterozygotes with plasma level of LDL-cholesterol ≥200 mg/dL and documented coronary heart disease† |

\*Stated LDL-cholesterol levels defined as the mean value of three serum samples obtained during a 2- to 4-week period after a 6-month trial of an American Heart Association Step II diet (or equivalent)<sup>314</sup> and maximum tolerated combination drug therapy defined as an adequate trial of drugs from at least two separate classes of hypolipidemic agents (eg, anionic exchange resins/bile acid sequestrants, HMG-CoA reductase inhibitors (statins), fibric acid derivatives, or niacin/nicotinic acid).

†Documented coronary heart disease defined as having one or more of the following:

- Prior documented myocardial infarction
- Prior coronary artery bypass graft surgery
- Prior percutaneous transluminal coronary angioplasty with or without atherectomy or coronary artery stent placement
- Significant angina pectoris documented by thallium or other heart stress test

LDL = low-density lipoprotein; HMG-CoA= 3-hydroxy-3 methylglutaryl co-enzyme A.

apheresis in heterozygotes.<sup>1,6</sup> The eligibility criteria for patients referred for LDL apheresis, according to the conditions of FDA approval of the devices, are presented in Table 43-11.<sup>317,318</sup> A regimen that combines medical therapy with LDL apheresis on a biweekly schedule can effectively lower LDL-cholesterol by 60% to 80% in otherwise treatment-resistant patients, improve the physical stigmata of hypercholesterolemia such as xanthomas and xanthelasma, improve myocardial perfusion and coronary artery patency, and favorably affect other markers of cardiovascular risk (eg, triglycerides, fibrinogen, homocysteine, C-reactive protein, adhesion molecules).<sup>312,319-324</sup> A summary of LDL apheresis systems is provided in Table 43-12.

**LDL Apheresis Devices Approved by the FDA**

The Liposorber LA-15 system<sup>317</sup> (Kaneka Pharma America, New York, NY) uses dextran sulfate bound to cellulose to selectively extract LDL-cholesterol from plasma. In this system, plasma is initially separated from the cellular components of blood by filtration through a disposable semipermeable polysulfone hollow fiber column, and the separated plasma is then perfused over a disposable adsorption column that contains 150 mL of dextran sulfate. The dextran sulfate has strong affinity for lipoproteins that contain apolipoprotein B [eg, LDL-cholesterol, VLDL-cholesterol, lipoprotein (a)] and adsorbs these from the plasma. The system has two adsorption columns connected in parallel to the tubing set, each capable of binding 7.1 g of LDL. One column is used to process 500 mL of plasma, and then the plasma is diverted to the second column, allowing for regeneration of the first column with a hypertonic saline solution that desorbs the bound lipoproteins from the dextran sulfate. The alternate use and regeneration of the two adsorption columns is controlled by a microprocessor.

The H.E.L.P. system<sup>318</sup> (B. Braun Medical, Bethlehem, PA) employs a 0.55-micron hollow fiber column to separate the plasma from the cellular elements of the blood. The plasma is

**Table 43-12.** LDL Apheresis Devices<sup>317,318,323-326</sup>

| Device                                               | Principle of Operation                                                                                                                                     | Anticoagulation      |
|------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------|
| *Liposorber LA-15 (Kaneka)                           | Electrostatic adsorption of apolipoprotein-B-containing lipoproteins to negatively charged immobilized dextran sulfate                                     | Heparin              |
| H.E.L.P. (B Braun)                                   | Precipitation of apolipoprotein-B-containing lipoproteins from acidified plasma by addition of heparin                                                     | Heparin              |
| *Direct Adsorption of Lipoproteins (DALI, Fresenius) | Adsorption of apolipoprotein-B-containing lipoproteins directly from whole blood perfused over negatively charged polyacrylate-coated polyacrylamide beads | Heparin and/or ACD-A |
| *Liposorber D (Kaneka)                               | Adsorption of apolipoprotein-B-containing lipoproteins directly from whole blood perfused over dextran sulfate covalently bound to cellulose               | ACD-A                |
| Therasorb-Ig (Miltenyi Biotech)                      | Adsorption of plasma lipoproteins using sheep antihuman-apolipoprotein B immobilized on Sepharose gel                                                      | Heparin              |
| Lipopak (Pocard)                                     | Adsorption of plasma lipoproteins using sheep antihuman-Lp(a) bound to Sepharose                                                                           | Heparin              |

\*Perfusion of plasma over negatively charged surfaces may result in excessive bradykinin generation and atypical or anaphylactoid-reactions in patients who are taking angiotensin-converting enzyme inhibitor drugs.<sup>327-329</sup>

acidified with 0.3M sodium acetate buffer and heparin is added to precipitate LDL-cholesterol. The LDL-cholesterol/heparin precipitate is filtered from the plasma using a 0.45-micron polycarbonate filter, excess heparin is adsorbed from the filtered plasma with a DEAE cellulose membrane filter, and the filtered plasma is then restored to physiologic pH by bicarbonate hemodialysis.

## Rheopheresis

Rheopheresis is a term that describes the therapeutic application of double filtration plasmapheresis to clinical disorders whose pathophysiology is believed to involve compromised microcirculatory blood flow.<sup>330</sup>

## Background

Rheopheresis is an apheresis technique in which whole blood is first pumped through a polyethylene hollow fiber membrane column [Plasmaflo OP-05W(L), Asahi Kasei Medical, Tokyo, Japan] which separates plasma from the formed elements of the blood. The plasma is then passed through a hollow fiber membrane column (SR-20 polysulfone, or AR-2000 cellulose diacetate, both from Asahi Kasei Medical) that filters large molecules, including LDL-cholesterol, fibrinogen,  $\alpha_2$ -macroglobulin, IgM, and von Willebrand factor from the plasma, resulting in a 50% to 60% decrease in their plasma levels after one plasma volume is processed.<sup>330,331</sup>

Clinical conditions in which rheopheresis therapy is under investigation include critical limb ischemia,<sup>332</sup> sudden sensorineural hearing loss,<sup>333</sup> and ischemic diabetic foot syndrome.<sup>334</sup> Rheopheresis has also been used to remove excess plasma phytanic acid in patients with Refsum's disease, an autosomal recessive disorder caused by mutations of phytanoyl/pristanoyl-CoA-hydroxylase.<sup>335</sup> But, arguably, the most important application of rheopheresis, at present, is in the treatment of dry age-related macular degeneration.<sup>331,336</sup>

## Rheopheresis in Dry Age-Related Macular Degeneration

In 2002 the World Health Organization considered 161 million people worldwide to be visually impaired, 37 million of whom were blind. Age-related macular degeneration (AMD) was identified as the primary cause of blindness in developed countries, accounting for at least 50% of blindness in North America, northern Europe, and Australia, and the third leading cause worldwide.<sup>337</sup> Based on demographic data from the 2000 US Census, over 50% of blindness among Americans of European heritage was a result of AMD, a proportion expected to increase as the American population ages.<sup>338</sup> The phrase "age-related" reflects the increasing risk of developing AMD after age 60. Over 13 million Americans above age 40 have signs or symptoms of macular degeneration, and 10% have the advanced "wet" stage of the disease.<sup>339,340</sup>

Current hypotheses regarding the pathophysiology of AMD consider diffusion of nutrients across Bruch's membrane, and the rheologic characteristics of blood flow through the choriocapillaris, to be deficient.<sup>341,342</sup> In part, these circumstances might result from a failure in the regulation of inflammation, as suggested by the strong association between macular degeneration

and inherited polymorphisms in the gene for complement factor H,<sup>342-347</sup> and the association of macular degeneration with elevations of C-reactive protein.<sup>348,349</sup> Therefore, rheopheresis would both improve microcirculatory flow in the choriocapillaris and across Bruch's membrane, and remove proinflammatory complexes from the blood, in the treatment of AMD.<sup>342</sup>

Based on promising early results from pilot studies in Germany<sup>350</sup> and the United States,<sup>341,342</sup> a properly powered prospective, randomized, multicenter, double-masked, placebo-controlled, Phase III trial of rheopheresis in dry AMD was conducted in 13 clinical centers in the United States.<sup>351,352</sup> The Multicenter Investigation of Rheopheresis for Age-Related Macular Degeneration (MIRA-1) trial randomly assigned patients to receive eight actual or sham rheopheresis treatments over a 10-week treatment period and tracked the change in best corrected visual acuity through 12 months after enrollment. At the conclusion of the study, 37% of study subjects in the treatment group, and 29% in the placebo group, were found to either not have met enrollment criteria or to have significantly violated the study protocol after enrollment, resulting in the absence of a statistically significant treatment effect in the final analysis of study outcome data.<sup>352</sup> Although a post hoc analysis that excluded the protocol violators indicated a significant treatment effect, the size of the study population evaluable for that analysis did not provide sufficient statistical power for conclusive interpretation of the results.<sup>352</sup> The study's sponsor, OccuLogix, Inc., a Toronto-based company, obtained permission from the FDA to conduct a follow-up study, RHEO-AMD, in order to definitively settle the issue of the efficacy of rheopheresis in AMD.<sup>353</sup> In 2007 OccuLogix suspended the RHEO-AMD trial because of financial considerations; thus, the future development of the technology for the treatment of dry AMD in the United States is uncertain.<sup>354</sup> Nonetheless, dry AMD is designated by ASFA as a Category P (pending) indication for double filtration plasmapheresis.<sup>1,6</sup>

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## Conclusion

The continued growth of therapeutic apheresis as a treatment option in diverse clinical conditions depends on an understanding of the pathophysiology of the disorders in question and the acquisition of evidence, from properly conducted clinical studies, of the efficacy of apheresis therapies in their management.

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## Disclaimer

The author has disclosed no conflicts of interest.

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# IV Hazards of Transfusion

## 44

## Overview of Hemovigilance

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The term “hemovigilance” has become widely used over the past decade to describe the systematic surveillance of adverse transfusion reactions and events, encompassing the whole transfusion chain and aimed at improving the safety of the transfusion process, from donor to recipient, “vein to vein.”<sup>1</sup> The term was coined in France in the early 1990s, has been developed and adopted internationally, and is now an integral part of transfusion practice.

Hemovigilance in its broader sense encompasses other important aspects of transfusion safety; surveillance of donors to ascertain the residual risks of transfusion-transmitted infections, traceability of transfused blood components, monitoring of blood utilization, epidemiology of transfused patients, and monitoring of the hazards of blood conservation. Compilation of this information, together with clinical assessment of transfused patients, has the potential to provide much-needed evidence of the outcomes, risks and benefits of blood transfusion, and transfusion alternatives.

Plasma derivatives may be included in hemovigilance or may be monitored according to pharmacovigilance. More recently, the concept of “biovigilance”<sup>2</sup> emphasizes the need for similar systems for donated tissues and stem cells.

This chapter focuses on surveillance systems, their design, what has been learned from them, how this information has been used to improve practice, and how information from other sources can contribute to the interpretation of these data.

### Requirements for an Effective Hemovigilance Scheme

A World Health Organization guideline on adverse event reporting and learning systems<sup>3</sup> emphasized that the effectiveness of an adverse event reporting system is measured not only by

accurate collection and analysis of data, but also by its use to make recommendations that improve patient safety. The guideline outlined the following core concepts:

- The fundamental role of patient safety reporting systems is to enhance patient safety by learning from failures of the health-care system.
- Reporting must be safe. Individuals who report incidents must not be punished or suffer other ill effects from reporting.
- Reporting is of value only if it leads to a constructive response. At a minimum, this entails feedback of findings from data analysis. Ideally, it also includes recommendations for changes in health-care procedures and systems.
- Meaningful analysis, learning, and dissemination of lessons learned require expertise and other human and financial resources. The agency that receives reports must be capable of disseminating information, making recommendations for changes, and informing the development of solutions.

These concepts are directly relevant to hemovigilance systems and are applicable both locally and nationally. At the hospital level, there is a need for a point of contact for clinical staff to report reactions and adverse events. This may be a transfusion practitioner or safety officer, hematologist, transfusion medicine specialist, or blood bank manager. There must be a “reporting and learning culture” within which events are used as learning opportunities, but also within a framework of professional competence and accountability; hemovigilance reporting should be an integral part of the risk management or clinical governance framework of the institution.

Hemovigilance reporting may be mandatory, as developed in France, or voluntary, as exemplified by the Serious Hazards of Transfusion (SHOT) scheme in the United Kingdom (UK). In both models, active participation is encouraged by educational feedback, and by confidence that centrally submitted data will be used effectively to build an evidence base for recommendations aimed at improvement. Confidentiality of patients, donors, and reporters must be ensured and the scheme must comply with legislative requirements for data protection.

At regional or national level, the hemovigilance scheme must be seen as impartial, independent, supportive, and professionally

credible. Data should be validated, analyzed, and reported within a predictable timeframe, and published in a format that can be used to support education and training. Active involvement and “ownership” by professional bodies will help to ensure that recommendations are incorporated into clinical practice.

Collection of complete data on adverse reactions and events requires local awareness and vigilance, and a robust reporting system that is easily understood and user friendly. The scope of the system must be clear to reporters, definitions must be unambiguous, and the system must be sufficiently flexible to respond to developments and changes in transfusion practice.

## The Scope of Hemovigilance

In discussing the scope of hemovigilance, the terms agreed upon by the Hemovigilance Working Group of the International Society of Blood Transfusion (ISBT) (P Robillard, personal communication) are used here.

An **adverse event** is an undesirable and unintended occurrence before, during, or after transfusion of blood or blood components that may be related to the administration of the blood or component. It may be the result of an error or an incident and it may or may not result in an adverse reaction in the recipient.

An **incident** is when the patient receives a blood component that did not meet all the requirements for a suitable transfusion for that patient, or was intended for another patient. It thus includes transfusion errors and deviations from standard operating procedures or hospital policies. It may or may not result in an adverse reaction.

A **near miss** is an error or deviation from standard procedure or policy that is discovered before the start of the transfusion.

An **adverse reaction** is an undesirable response or effect in a patient temporally associated with the administration of blood or blood components. It may be the result of an incident or an interaction between the recipient and blood, a biologically active material.

It is then necessary to determine the severity of the reaction, and the imputability, ie, the degree to which, on completion of investigation, the transfusion is considered to be the cause of the reaction. The ISBT Working party scales of severity and imputability may be found in Appendix 44-1 at the end of this chapter.

Adverse reactions occurring during or within 24 hours after transfusion are likely to be recognized as being transfusion related, but the longer the interval between the transfusion and the reaction, the less likely the reaction is to be identified and the causation determined. Continuing education of clinicians responsible for transfused patients is needed to improve awareness of transfusion reactions and ensure that they are investigated and reported appropriately.

## What Should Be Reported?

### Adverse Reactions

As will be described in the following section, national hemovigilance systems vary in their scope and in the type and severity of adverse

reactions they wish to have reported to them. There is some commonality; all established systems would expect to receive reports of severe allergic or anaphylactic reactions, acute and delayed hemolytic reactions, transfusion-transmitted infections (TTIs), transfusion-related acute lung injury (TRALI), transfusion-associated graft-vs-host disease (TA-GVHD), and posttransfusion purpura (PTP). These severe reactions are uncommon, and a hemovigilance scheme such as SHOT that restricts its scope to serious reactions receives approximately 10 such reports per 100,000 blood components issued by the blood services.<sup>4</sup> Most schemes invite reports of transfusion-associated circulatory overload (TACO), the frequency and importance of which are increasingly widely recognized.<sup>5</sup>

Many hemovigilance systems also encompass less severe reactions, eg, febrile nonhemolytic transfusion reactions (FNHTRs), and mild allergic reactions presenting with only mucocutaneous symptoms and signs. Some systems also receive reports of alloimmunization, ie, the finding of a new clinically significant red cell antibody in a previously transfused patient but without clinical or laboratory signs of hemolysis. Minor reactions are common and, although they may be distressing for the patient, they require minimal clinical intervention. However, they produce a large workload if they are required to be reported to the hemovigilance system.<sup>6</sup> It may be argued that such comprehensive reporting is excessively time-consuming and cannot be clinically justified; however, because the observed rate of FNHTRs is known to be 0.5% to 1% of red cell transfusions overall,<sup>7</sup> the number of these reports from an individual institution can provide a useful indicator of active participation in hemovigilance, and enable benchmarking between hospitals (P Robillard, personal communication).

Although clear definitions of adverse reactions are necessary to ensure consistent reporting and enable comparisons, it must also be possible to report previously unrecognized complications that may not fit into one of the recognized definitions.

### Adverse Incidents

These events, sometimes referred to as “incorrect blood component transfused” (IBCT), result from avoidable system failures throughout the transfusion chain. Experience from the SHOT scheme over a period of 8 years has indicated that the observed rate of such events is 7:100,000 blood components issued, with a fatal outcome in 0.7:100,000.<sup>4</sup> Other studies, using different methods, have calculated an error rate of 1:19,000<sup>8</sup> and a risk of death caused by an ABO-incompatible transfusion of 1:1,800,000 allogeneic red cell transfusions.<sup>9</sup>

Detailed analysis of these incidents reveals that in approximately 50% of events there are multiple errors in the transfusion process, that approximately 70% of errors occur in clinical areas (the most frequent error being failure to ensure at the bedside that the right blood is being given to the right patient), and that 30% happen in hospital laboratories.<sup>10</sup>

### Near Misses

Near misses include those occasions an error was detected, either by chance or as a result of quality checks, and an incident



prevented. As in other fields (eg, aviation), the study of near misses in health care can highlight high-risk areas and may also identify effective recovery systems. Near misses are approximately 10 times more frequent than incidents, and again, the work required for comprehensive reporting must be balanced against the potential benefit. These events may enable identification of previously under-recognized errors. For instance, “wrong blood in tube” (ie, pretransfusion blood sample taken from the wrong patient or labeled with the wrong patient’s details) is an infrequent cause of IBCT. However, sampling errors are, in fact, common, accounting for 56% of near-miss reports.<sup>4</sup>

A structured system for medical event reporting in transfusion medicine (MERS-TM) can identify high-risk areas and processes requiring system change.<sup>11</sup> A 3-year pilot project of near-miss reporting in Ireland using this approach demonstrates the value of such an approach.<sup>12</sup>

### Blood Management

With the impending threat of blood supply shortages and increasing awareness of the known and unknown risks of transfusion, much emphasis has been placed on alternatives to allogeneic transfusion and conservative transfusion policies. If hemovigilance is to provide a true picture of the risks and benefits of transfusion, then the hazards of blood conservation should also be included. Few systems currently record the risks of blood recovery and reinfusion or adverse reactions to pharmacologic agents.

Transfusion safety encompasses the appropriate use of blood components, but requires assessment by clinical audit.<sup>13</sup> It is not within the scope of hemovigilance.

### Biovigilance

A number of commonalities can be recognized between blood transfusion and transplantation of tissues and hemopoietic stem cells. Infectious complications, system failures, and other adverse events may occur in both situations. Although the clinical situations and stakeholder groups may differ, the basic principles are the same, and experience in hemovigilance is of value when designing adverse event reporting and learning systems for tissues and cells. In Europe, legislation for tissues mirrors that for blood, and the development in the United States of new and comprehensive systems for “biovigilance” is awaited with great interest.<sup>2</sup>

### Passive Reporting vs Active Surveillance

Passive reporting systems, such as those described here, capture adverse events occurring within a short time following transfusion, provided clinical suspicion is high and an effective reporting mechanism is in place. Active surveillance of transfusion recipients may reveal a different picture, as illustrated by the difference in the incidence of TRALI reported by SHOT as 0.6 per 100,000 blood components issued<sup>4</sup> but reported by a single hospital with high clinical awareness as 1 in 8000 blood components issued.<sup>14</sup> Similarly, a prospective study of bedside errors found

major errors (including misidentification) in 15 of 3485 units transfused.<sup>15</sup>

Recognition of delayed complications may also require a different approach. Evidence for transfusion transmission of variant Creutzfeld-Jakob disease (vCJD) has been obtained only by rigorous implementation of active surveillance, involving the tracing of recipients of blood components from donors who were subsequently identified as developing vCJD, and retrospective tracing of blood donors who provided components to patients who developed vCJD.<sup>16</sup>

### Traceability

Two-way traceability, from donor to recipient and reverse, is essential for good manufacturing practice and for hemovigilance, and is a requirement of the European Union (EU) Blood Directive<sup>17</sup> and the United States Food and Drug Administration (FDA) regulations.<sup>18</sup>

The ISBT 128 identification system for blood components provides a global standard for identification of human blood, tissue, and organ products to individual donors across international borders and disparate health-care systems, and has been designed and perfected over a 12-year period.<sup>19</sup> Blood collectors must be able to trace blood from the donor, through component processing and testing, to the point of issue to the hospital blood bank; thereafter, the hospital blood bank or transfusion service is responsible for documenting that a specific blood component was transfused to an individual recipient, or its final fate if not transfused.

Forward immediate traceability is essential if a donor becomes ill with a TTI within the shelf-life of the donation, when prompt treatment of the recipient may preempt the development of clinical illness. In the case of a blood pack fault or test kit failure, a robust traceability system will facilitate rapid recall of components and minimize risk to patients. In the longer term, full two-way traceability is essential for look-back, eg, when a new screening test is introduced<sup>20</sup> or a new transfusion-transmissible agent recognized.<sup>21</sup>

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## Models of Hemovigilance in Different Countries

### History

Pharmacovigilance was the first surveillance system in the arena of health care, covering medicinal products of all kinds. Hemovigilance, as a separate surveillance system, was first implemented in France in 1994, as required by the updated French regulation on blood in a response to a “blood scandal.” In the UK, anticipation of forthcoming European legislation, together with concerns regarding transfusion safety, led to the establishment of SHOT in 1996.

By the end of the 1990s, the concept of hemovigilance was developing across Europe but there was a lack of commonality of definitions, terminology, structure, and scope of reporting. In many countries, development was hindered by organizational

difficulties, lack of funding, unclear mandates, undefined responsibilities, inadequate training, and inability to make changes in response to reported findings.

At the turn of the millennium, hemovigilance was established and functioning in several countries across Europe, although not all were national systems. In some countries hemovigilance was required by law, in some it was officially established but not functioning, and in still others it was established as a voluntary system. Table 44-1 shows the situation in the 15 “old” member states of the EU in 2004.

The two national systems in France and the UK represented polarities in European hemovigilance. In France, the hemovigilance system was from the start centralized and nationwide, with a legal requirement for written notification of each and every side effect in relation to a blood transfusion (grades 1 to 4). Between 7000 and 7500 reports are generated annually, with an estimated 2.5 million blood components transfused every year in France.<sup>6</sup>

In the UK, where hemovigilance was designed as a national, voluntary, professionally led scheme covering transfusion reactions of grades 2 to 4, and IBCT regardless of outcome, the number of notifications is much lower than in France, with 2630 reports received in 8 years, 70% of which were IBCT.<sup>4</sup>

Subsequently, hemovigilance systems were established in nearly all of the 15 “old” member states of the EU.

The Republic of Ireland contributed to SHOT until 1998, when the Irish National Haemovigilance Office was established, again in response to “blood scandals” relating to hepatitis C virus. The Irish program is largely modeled on SHOT, and has included a pilot study of near misses.<sup>12</sup>

In Germany, blood components are considered by law to be medicinal products. As such they fall under German Drug Law, and pharmacovigilance applies to component production.

For the clinical aspects of transfusion (the users of blood components), hemovigilance applies and there is a major challenge in linking these surveillance activities together.

In Austria, the situation is comparable to Germany, although surveillance of collection, preparation, and transfusion of blood components are part of hemovigilance.

In the Benelux countries (Belgium, the Netherlands, and Luxembourg), small surveillance systems were established; initially these were fragmented and lacking in coordination. The Transfusion Reactions in Patients (TRIP) scheme in the Netherlands is now well established and produced its first annual report in 2003.<sup>22</sup> A major contribution of the Benelux countries lies in their leading role in creating and developing the European Haemovigilance Network (EHN).

In Denmark, hemovigilance was initially established on a local basis, until the creation of the Danish Registration of Transfusion Risks (DART).<sup>23</sup> The Danish system was the first to introduce structured donor hemovigilance, and the importance of donor adverse reactions was emphasized from the outset. The Danish model has largely influenced donor hemovigilance in other European countries, as now required by the EU Blood Directives.

In Greece, there is a long tradition of hemovigilance and, despite the complex structure of the Greek transfusion system because of geographic and political factors, these surveillance activities have generated interesting data.<sup>24</sup>

In Spain, impressive progress has been made recently, showing that, even in a complicated political environment, professionals from the field who have a strong will to bring hemovigilance forward can achieve significant results in a short period.

For the 12 “new” member states (joining the EU in May 2004), little is known about national hemovigilance activities, but this will change quite rapidly as these countries work toward compliance with the EU Blood Directive.

**Table 44-1.** Hemovigilance in Europe in 2004 before Implementation of the European Union Blood Directive

| Member State   | Status of Hemovigilance | Mandatory or Voluntary | Responsible Organization                                     |
|----------------|-------------------------|------------------------|--------------------------------------------------------------|
| Austria        | Started in 2003         | M                      | Österreichisches Bundes-Institut für Gesundheit              |
| Belgium        | Established/functioning | M                      | Red Cross                                                    |
| Denmark        | Established/functioning | V                      | Danish Registry for Adverse Reactions in Transfusion         |
| Finland        | Established/functioning | V                      | Red Cross                                                    |
| France         | Established/functioning | M                      | Agence Française de Sécurité Sanitaire des Produits de Santé |
| Germany        | Established/functioning | M                      | Paul Ehrlich Institute                                       |
| Greece         | Established/functioning | V                      | Hellenic Co-ordinating Haemovigilance Centre                 |
| Ireland        | Established/functioning | V                      | National Haemovigilance Office                               |
| Italy          | In development          | V/M                    | Instituto Superiore de Sanita + Regions                      |
| Luxembourg     | Established/functioning | V                      | Red Cross + Ministry of Health                               |
| Netherlands    | Started in 2003         | V                      | Transfusion Reactions in Patients                            |
| Portugal       | In development          | M?                     | Instituto Portugues do Sangue + Ministry of Health           |
| Spain          | Started in 2004         | V                      | Programa Estatal de Hemovigilancia + Regulator               |
| Sweden         | In development          | V/M                    | Swedish Association for Transfusion Medicine + Hospitals     |
| United Kingdom | Established/functioning | V                      | Serious Hazards of Transfusion                               |

## The EU Blood Directives and the Future of European Community Hemovigilance

### Blood Directives

The key role of hemovigilance in blood transfusion safety is reflected in the EU Blood Directive 2002/98/EC.<sup>17</sup> This Directive of the European Parliament and of the Council of Europe sets standards of quality and safety for the collection, testing, processing, storage, and distribution of human blood and blood components and amends Directive 2001/83/EC. An entire chapter is dedicated to hemovigilance, and encompasses traceability and notification of serious adverse events and reactions.

According to the Directives on blood, hemovigilance is defined as “a set of organized surveillance procedures relating to serious adverse or unexpected events or reactions in donors or recipients, and the epidemiologic follow-up of donors.” It is stated that “it is important to introduce a set of organized surveillance procedures to collect and evaluate information on the adverse or unexpected events or reactions resulting from the collection of blood or blood components in order to prevent similar or equivalent events or reactions from occurring thereby improving the security of transfusion by adequate measures. To this end a common system of notification of serious adverse events and reactions ... should be established in member states.”

The legal framework on blood at the European Community level comprises four Directives:

- 2002/98/EC (Directive on principles and organization)
- 2004/33/EC (Directive on donations, donors, and blood components)
- 2005/61/EC (Directive on traceability and notification—hemovigilance)
- 2005/62/EC (Directive on a quality management system).

The Directives lay down standards of quality and safety of human blood and of blood components, in order to ensure a high level of human health protection. These are minimum standards, and do not require harmonization of activities related to blood transfusion in the member states of the EU.

In the context of blood matters, including hemovigilance, it is important to understand that the European Treaty (treaty of Amsterdam, establishing the European Community) sets limits in Article 152 that restrict the Directives on blood to collection and testing of human blood and blood components, whatever their intended purpose, and their processing, storage, and distribution when intended for transfusion. However, the Directives do not apply to clinical activities.

Hence, although hemovigilance (including rapid alert) should encompass the whole blood chain from donor to recipient and vice versa, including production and clinical use, current legislation can cover only the producers (the blood establishments) and to some extent the hospital blood banks. It cannot include users and their clinical activities, which are the exclusive responsibility of the individual member states. This obviously creates a complex situation in Europe with regard to hemovigilance systems.

In Chapter V of Directive 2005/61/EC, on hemovigilance, there are legally binding requirements for traceability (Article 14) and for the notification of serious adverse events and

reactions (Article 15). In the definition of “hemovigilance” it is stated that the organized surveillance procedures should relate to serious adverse or unexpected events or reactions in donors or recipients, and to the epidemiologic follow-up of donors.

The Directive requires reporting of only serious adverse or unexpected events or reactions.

A “*serious adverse event*” is defined in the Directive as any untoward occurrence associated with the collection, testing, processing, storage and distribution of blood and blood components that might lead to death or life-threatening, disabling, or incapacitating conditions for patients or that results in, or prolongs, hospitalization or morbidity.

A “*serious adverse reaction*” is defined in the Directive as an unintended response in a donor or patient associated with the collection or transfusion of blood or blood components that is fatal, life-threatening, disabling, incapacitating, or that results in, or prolongs, hospitalization or morbidity.

In the definition of “hemovigilance” it is required that organized surveillance cover both donors and recipients. The principle of subsidiarity allows other issues to be determined by individual EU member states, which may adopt regulations in addition to the provisions in the Blood Directives. However, the Directives must be transposed into national legislation by the member states.

### European Hemovigilance

Some years ago the need for EU-wide cooperation was anticipated when several countries decided to create a network for hemovigilance. The EHN was founded in 1998 by representatives from Belgium, France, Luxembourg, the Netherlands, and Portugal; these countries were later joined by Austria, Denmark, Finland, Greece, Ireland, Malta, Slovenia, Spain, and United Kingdom. The boundaries were more recently extended to include Canada, Croatia, Iceland, New Zealand, Norway, Singapore, and Switzerland. Further expansion is likely in future.

The EHN is conceptually a bottom-up organization of professionals from the field and includes different specialties: transfusion medicine, hematology, internal medicine, and civil servants.

The aim of the EHN is to develop and maintain in Europe a common structure with regard to safety of blood and blood components and hemovigilance in blood transfusion and transfusion medicine. To this end, the EHN pursues the following objectives:

- Favor exchange of valid information between members.
- Increase rapid alert and early warning between members.
- Encourage joint activities between members.
- Undertake educational activities relating to hemovigilance.

To fulfill these objectives, the EHN has:

- Developed and maintained a website ([www.ehn-org.net](http://www.ehn-org.net)).
- Established an on-line system for rapid alert and early warning.
- Worked toward standard definitions relevant to hemovigilance, in collaboration with ISBT.
- Initiated standardization of processes and forms.
- Started to compile, analyze, and compare data generated by the national hemovigilance systems.
- Organized annual European Haemovigilance Seminars.

The Rapid Alert system (RAS) was established and has been maintained by the EHN as a high priority from the outset, providing a framework for the international transmission of information on unexpected adverse reactions or equipment defects. It is especially useful for those countries where no previous system existed. A designated official contact person (OCP) in each country is responsible for the transmission and receipt of information, and coordinates with the blood services regarding appropriate corrective or preventive action to maintain or improve safety.

The EHN-RAS is a network of OCPs who are nominated or nationally agreed-upon individuals, responsible for entering, retrieving, and filtering information, and onward transmission if appropriate. The EHN-RAS has been used to disseminate information on newly recognized transfusion reactions (eg, red-eye syndrome), adverse reactions in donors (eg, allergic or anaphylactic reactions associated with apheresis devices), defects in disposables, problems with equipment or information technology, and deficiencies in reagents.

In the first 5 years more than 30 alerts were launched and disseminated to participating countries and organizations, and the RAS has developed with increasing cooperation of manufacturers. There remains scope for further development.

In Europe it was recognized quite early that hemovigilance has an important role. With the impact of the Directives on blood, national hemovigilance systems in Europe will converge to some degree, but will remain autonomous. The development of EHN has demonstrated that international cooperation at a professional expert level is possible, and can be effective in improving the safety and quality of blood transfusion. It is encouraging that newly established hemovigilance schemes worldwide are seeking to maintain and further develop established professional links, and EHN works closely with the Haemovigilance Working Group of the ISBT.

## Development of Hemovigilance Worldwide

An international forum in 2006 indicated the extent to which hemovigilance is becoming an integral and important part of transfusion practice worldwide.<sup>25</sup> Twenty-four countries responded to a survey; all but a few had a full hemovigilance system in place. The Japanese Red Cross has had a comprehensive hemovigilance system since 1993, and data are now coming from New Zealand, Russia, the Czech Republic, Slovakia, Poland, South Africa, and Brazil, as well as established systems in Europe, Scandinavia, and Canada. Work is in progress to develop hemovigilance in the US, Australia, and North Africa.

Comparisons of observed incidence of adverse reactions between countries with different practices can assist risk/benefit analysis and policy decision making. For example, in Norway, where there is 100% use of solvent/detergent-treated plasma (Octaplas, Octapharma, Lachen, Switzerland), the frequency of adverse events observed with plasma is low in comparison with that of other countries using Fresh Frozen Plasma (FFP).<sup>25</sup>

If comparative data are to be useful, like must be compared with like or, at the very least, differences in methodology must be recognized and understood. To this end the Haemovigilance Working Group of the ISBT is developing consensus definitions of adverse incidents in recipients and donors.

Denominator data are needed for interpretation of hemovigilance findings in the context of transfusion practice. The number of reported incidents is meaningful only when expressed against a denominator of blood components transfused. However, in many countries, including the UK, the only accurate figure available is the number of components issued by the blood collectors, with little or no reliable data on the number of components actually transfused, the number of transfusion episodes, or transfused patients. The question also arises as to whether the denominator should include blood components issued to hospitals that do not report incidents, as is done by SHOT in the UK, or should be restricted to components issued to actively participating hospitals, as in Canada (P Robillard, personal communication).

Epidemiologic studies are beginning to cast light on the clinical situations in which blood is used and the demographic profile and survival of transfused patients.<sup>26-28</sup> This will enable the future development of more sophisticated denominator data, identification of high-risk patients and situations, and estimation of the risks of transfusion in an individual patient.

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## Hemovigilance Related to Blood Donors and Blood Donation

Systematic surveillance of the first part of the transfusion chain—the collection of blood from the donor—is an essential element of hemovigilance, and aims to secure and improve the safety of both the donor and the recipient. It should include registration of unexpected adverse events in whole blood and component donors and the action taken as a result. These events may be adverse reactions or complications resulting from donation, or adverse events related to the selection and management of donors, which may directly harm the donor or influence the quality of the product, thereby harming the recipient.

Provision of information about the risks of donation based on evidence from surveillance, together with good clinical management of any complications, indicates a high professional standard of the blood collection facility and its care of the well-being of the donors. This, in turn, will improve donor confidence and satisfaction, making it more likely that the donor will return, thus benefitting the national supply of blood.<sup>29,30</sup> In 2004, awareness of the importance of donor complications was raised by presentations of preliminary data from a pilot study.<sup>31,32</sup> A joint working group from the ISBT and EHN was established, and has proposed a classification and a set of definitions of complications related to blood donation (Appendix 44-2), to form the basis for a registry. It is hoped that the use of this registry will enable development of an evidence base to support improvements in donor care and selection.

### Complications Related to Regular Whole Blood Donation

Data on the occurrence of these complications are sparse, because most hemovigilance systems have not hitherto included donor adverse events. Large studies have been reported from single blood centers in the United States and in Greece<sup>33-38</sup>; comprehensive national data have been reported from Denmark<sup>39</sup>; and data collected with the use of the definitions agreed-upon have been presented at several meetings (Table 44-2).

The rate of reported donor complications has varied according to the severity and range of reactions included. Preliminary data registered according to the ISBT classification suggest that the overall rate of complications may be as high as approximately 1 per 100 donations. The most common complications related directly to the bleeding process are vasovagal reactions (2/3) and injuries of soft tissues, vessels (1/3), nerves, or tendons caused by the inserted needle.

#### Vasovagal Reactions

Vasovagal reaction is the most common donor complication (488 per 100,000 donations). The predominant symptoms are general discomfort, weakness, anxiety, dizziness, nausea, sweating, vomiting, pallor, and hyperventilation, associated with hypotension and bradycardia. The two last symptoms are essential for the diagnosis.

Most vasovagal reactions are mild and transient, but some donors may lose consciousness (vasovagal syncope). In the more severe cases this may result in an accident, if the donor faints and falls, or may be associated with convulsions and incontinence. It is important to reassure the donor that these reactions do not indicate a predisposition to true epilepsy.

Vasovagal reactions are generated by the autonomic nervous system stimulated by psychologic factors, and by the volume of blood removed relative to the donor's total blood volume. Therefore, donor age, weight, and donation status have been found to be significant variables.<sup>40</sup> The risk can be reduced by fluid replacement,<sup>41</sup> and by careful management of the donors during their stay in the blood facility.

Some vasovagal reactions (~10%)<sup>39</sup> occur after the donor has left the donation area, so-called delayed reactions. These reactions are potentially dangerous, as the donor may be at risk of a serious accident, and occasional deaths have been reported anecdotally.

In order to lower the risk of delayed reactions, and of serious outcome of these, it is essential to ensure that the donor feels completely well before leaving the donation area. Many facilities permanently defer donors following a severe delayed vasovagal reaction. Donors with hazardous occupations where they or others could be put at risk (eg, pilots) should not return to work within 24 hours of donation.

#### Hematomas

Hematoma is the second most common complication related to blood donation (275 per 100,000 donations). The symptoms are bruising, swelling, and pain at the venipuncture site.

A hematoma may occur if the needle punctures small arterial vessels, or if blood leaks from the vein during or after venipuncture. Blood in the soft tissues behind the biceps tendon will initially spread behind the tendon and may not produce any

visible swelling or pain. As the hematoma increases in size, it tracks along the blood vessels, nerves, and tendons to the forearm, and may cause paresthesiae in the fingers because of compression or irritation of the median nerve.

Accidental puncture of a large artery in the antecubital fossa carries a high risk of a hematoma and of delayed bleeding, and other rare but serious complications (Appendix 44-2). Donor care staff should be trained to recognize and correctly manage arterial puncture, so as to avoid extremely rare, but very severe, complications such as compartment syndrome of the forearm.

Injury to the median nerve in the antecubital fossa accounts for only 8% of all immediate adverse reactions, but gives rise to nearly all serious long-term complications.<sup>39</sup>

In approximately one-third of these cases the median nerve is directly damaged by the venipuncture needle.<sup>39</sup> The donor may experience immediate severe shooting pain, radiating down the forearm, sometimes associated with paresthesiae in the median nerve distribution of the hand. Indirect nerve injury may be caused by pressure from an increasing hematoma, with symptoms of pain and paresthesiae developing later.

Anatomical studies have shown marked individual variation in the arrangement of nerves and blood vessels in the antecubital fossa, and injuries may occur despite good venipuncture technique.<sup>42</sup> It is essential however that venipuncturists are familiar with normal anatomy of the region and are trained in correct techniques of needle insertion, together with prompt recognition and correct management of complications. To reduce the risk of direct nerve injury, the needle should be inserted only once and, if unsuccessful, no further attempts should be made.

Hematoma should be managed by stopping the donation immediately if the donor complains of symptoms, applying pressure to the venipuncture site, and recommending that the donor rest the arm and avoid manual work for 24 hours. The donor should return to the blood center or seek medical treatment if there is persistent bleeding from the venipuncture site or if the swelling increases.

### Long-Term Effects of Regular Whole Blood Donation and Apheresis

The surveillance of donors should also include the long-term effects of regular whole blood donation and repeated apheresis. Those of most concern are depletion of iron, calcium, and plasma proteins. The surveillance of repeat donors of whole blood should include at least the measurement of the hemoglobin concentration on each occasion. Programs of intensive plasmapheresis should include monitoring of plasma albumin concentrations.<sup>43</sup>

### Learning from Hemovigilance

Effective hemovigilance requires not only accurate and complete collection of data, but also interpretation of the data in the context of what is known of transfusion epidemiology and practice, and use of the data to improve patient safety, both locally and nationally.

**Table 44-2.** Occurrence of Complications Related to Donation of Whole Blood

| Occurrence Shown as Number of Cases/100,000 Donations |                                                      | Donor Complications (all grades) |          |              |       | Donor Complications (serious) <sup>†</sup> |          |              |       |
|-------------------------------------------------------|------------------------------------------------------|----------------------------------|----------|--------------|-------|--------------------------------------------|----------|--------------|-------|
| Study                                                 | Donations Surveilled (comments)                      | Vasovagal Reaction               | Hematoma | Nerve Injury | Total | Vasovagal Reaction                         | Hematoma | Nerve Injury | Total |
| Newman <sup>33</sup>                                  | 1,000 (interview 3 weeks after donation)             | 6400                             | 1700     | 900          | 9000  |                                            |          |              |       |
| Newman <sup>33</sup>                                  | 1,000 (information obtained at donation)             | 2100                             | 324      | 16           | 2440  |                                            |          |              |       |
| Khan <sup>35</sup>                                    | 5,536 (random)                                       | 1210                             | nd       | nd           |       |                                            |          |              |       |
| Newman <sup>34</sup>                                  | 949 (random)                                         | 2600                             | nd       | nd           |       |                                            |          |              |       |
| Newman <sup>32</sup>                                  | 419,000 (random)                                     | nd                               | nd       | 16           |       | nd                                         | nd       | 5            |       |
| Zervou <sup>37</sup>                                  | 12,173 (one center)                                  | 879                              | nd       | nd           |       |                                            |          |              |       |
| Sorensen <sup>38</sup>                                | 2,575,246 (national register* serious complications) |                                  |          |              |       | 7                                          | 1        | 11           | 19    |
| Sorensen <sup>38</sup>                                | 41,274 (county register* all complications)          | 478                              | 274      | 70           | 822   |                                            |          |              |       |
| Caffrey <sup>‡</sup>                                  | 2,332,439 (national data,* England and N.Wales)      | 1399                             | 36       | 10           | 445   | 9                                          | 3        | 2            | 14    |
| Hauser <sup>40</sup>                                  | 2,196,900 (national data, France)*                   |                                  |          |              |       | 5                                          | 1        | 0.4          | 6     |
| Okazaki <sup>†</sup>                                  | 3,758,712 (national data, Japan)*                    | 736                              | 93       | 7            | 836   | 5                                          | 2        | 3            | 10    |

\*Data collected in accordance with *Standard for Collecting and Presentation of Data on Complications Related to Blood Donation* (Version 2007).<sup>†</sup>Complications with symptoms that lasted for more than a year or that needed medical treatment were graded as serious.<sup>‡</sup>Personal communications.

Early SHOT data focused attention on avoidable system failures in the transfusion chain, with the potential for serious patient harm. Acute intravascular hemolysis caused by major ABO incompatibility is the most feared outcome, but transfusion errors can cause patient harm in other ways, such as failure to provide blood of the correct specification for the patient, eg, irradiated cellular components for immunosuppressed patients at risk of TA-GVHD, or D sensitization of D-negative females of childbearing potential. Volume overload or unnecessary exposure to blood components can result from 1) incorrect decision making; 2) prescribing errors such as volume miscalculation; or 3) transfusing based on a misleading hemoglobin level from a sample diluted by intravenous infusion, from the wrong patient, or from a laboratory result that is incorrect or wrongly documented.

Over a 10-year period, during which time 30 million blood components were issued from the UK blood services, SHOT received 3770 reports, of which 2717 (72%) were of IBCT (see Table 44-3 and Fig 44-1). Ninety-five percent of these patients survived with no serious effects, but 24 deaths were attributed wholly or in part to avoidable transfusion errors, and 100 patients suffered major morbidity.<sup>44</sup>

Detailed analysis of these incidents is necessary if lessons are to be learned from them. For example, the SHOT questionnaire

on IBCT elicits the stage(s) of the transfusion chain where the error(s) occurred, and has thus been able to identify that 70% of errors occur in clinical areas, the most frequent error being failure to correctly perform the pretransfusion patient identification check at the bedside.<sup>10</sup> Approximately 30% of errors occur in hospital transfusion laboratories<sup>10</sup> and detailed knowledge of the circumstances can help to support improvements in standards of practice.

Errors in blood sampling for pretransfusion testing are usually detected in the laboratory if the patient is previously known. Data on near misses from SHOT and from the National Haemovigilance Office in Ireland<sup>12</sup> have revealed the true frequency of such errors and highlighted the importance of correct patient identification at the point of blood sampling.

Information on the time and place of transfusion, and the level of staff involved, is useful—not to apportion blame, but to target education and training and to direct efforts toward improving standards of practice. Such detail will be forthcoming only if reporters are assured that information will be used positively and constructively, and in total confidence.

Reports to SHOT, when interpreted in the context of epidemiologic data, suggest that night-time transfusion carries an increased risk<sup>45</sup> compared to those administered during the day

**Table 44-3.** SHOT Cumulative Morbidity/Mortality 1996 to 2006

|                                                                            | All Causes   | IBCT        | ATR*       | HTR*       | PTP       | TA-GVHD   | TRALI      | TTI       |
|----------------------------------------------------------------------------|--------------|-------------|------------|------------|-----------|-----------|------------|-----------|
| Death definitely attributed to transfusion (imputability 3)                | 47           | 7           | 2          | 6          | 1         | 13        | 8          | 10        |
| Death probably attributed to transfusion (imputability 2)                  | 15           | 4           | 4          | 1          | 0         | 0         | 6          | 0         |
| Death possibly attributed to transfusion (imputability 1)                  | 47           | 13          | 7          | 1          | 1         | 0         | 25         | 0         |
| <b>Subtotal 1</b>                                                          | <b>109</b>   | <b>24</b>   | <b>13</b>  | <b>8</b>   | <b>2</b>  | <b>13</b> | <b>39</b>  | <b>10</b> |
| Major morbidity† probably or definitely attributed to transfusion reaction | 315          | 100         | 17         | 29         | 13        | 0         | 118        | 38        |
| Minor or no morbidity as a result of transfusion reaction                  | 3324         | 2582        | 387        | 280        | 31        | 0         | 38         | 6         |
| Outcome unknown                                                            | 15           | 11          | 3          | 0          | 0         | 0         | 0          | 0         |
| <b>Subtotal 2</b>                                                          | <b>3654</b>  | <b>2693</b> | <b>107</b> | <b>310</b> | <b>44</b> | <b>0</b>  | <b>156</b> | <b>44</b> |
| <b>TOTAL</b>                                                               | <b>3763‡</b> | <b>2717</b> | <b>420</b> | <b>318</b> | <b>46</b> | <b>13</b> | <b>195</b> | <b>54</b> |

\*The HTR category was formerly DTR (delayed transfusion reactions); from 2006, all hemolytic reactions are included in HTR.

†Major morbidity is classified as the presence of one or more of the following:

- Requirement for intensive care and/or mechanical ventilation.
- Dialysis or renal impairment.
- Minor hemorrhage from transfusion-induced coagulopathy.
- Intravascular hemolysis.
- Potential D sensitization in a female of childbearing potential.

‡Excludes seven unclassified cases from 1998/1999.

SHOT = serious hazards of transfusion; IBCT = incorrect blood component transfused; ATR = acute transfusion reaction (included acute hemolytic reactions until 2006); HTR = hemolytic transfusion reaction (pre-2006 this included only delayed hemolytic transfusion reactions); PTP = posttransfusion purpura; TA-GVHD = transfusion-associated graft-vs-host disease; TTI = transfusion-transmitted infection.

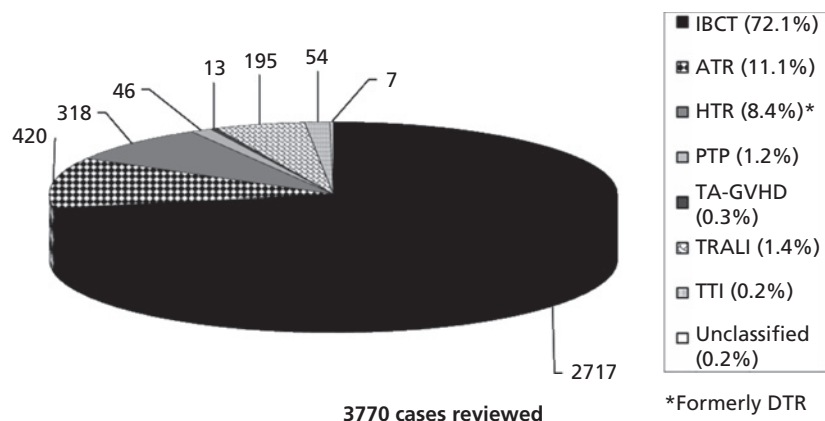


**Figure 44-1.** Analysis of reports to SHOT over the 10-year period 1996.

SHOT=Serious hazards of transfusion; IBCT=Incorrect blood component transfused; ATR=Acute transfusion reaction (included acute hemolytic reactions until 2006); HTR=Hemolytic transfusion reaction (pre-2006 this included only delayed hemolytic transfusion reactions); PTP=Post transfusion purpura; TA-GVHD=Transfusion-associated graft-vs-host disease; TTI=Transfusion-transmitted infection.

and that transfusion to pediatric patients is more likely to result in an adverse outcome than adult transfusion.<sup>46</sup>

Successive SHOT reports have led to a number of national safety initiatives including professional guidelines on blood administration,<sup>47</sup> a nursing guideline,<sup>48</sup> a Safer Practice Notice from the National Patient Safety Agency relating to bedside pre-transfusion checks,<sup>49</sup> and a Quality Standard on transfusion.<sup>50</sup>

Hemovigilance data can be enormously valuable for monitoring changes in practice, such as the implementation of universal leukocyte reduction, which in France was noted to result in a reduction in the incidence of FNHTRs,<sup>9</sup> and in the UK was found to reduce, but not eliminate, TA-GVHD and alter the pattern of PTP.<sup>51</sup> Hemovigilance data have also been used to monitor measures to reduce the risk of bacterial contamination of platelets, and strategies to reduce the incidence of TRALI.

Cumulative SHOT data on transfusion-related mortality and morbidity<sup>4</sup> revealed the importance of TRALI as the leading cause of such outcomes. These findings are consistent with those from other countries, although hemovigilance data almost certainly underestimate the risk of this controversial and complex complication of transfusion. In institutions with a high index of suspicion of TRALI, its incidence has been estimated as 1:2000 to 1:8000 plasma-containing components.<sup>14,52</sup> Reports of TRALI to SHOT remained level at an average of 14 (range 11-19) per year for the first 5 years, but rose to 26 in 2001/2002, and to 36 in 2003, with increasing awareness of the condition. This led, in 2003, to a policy implemented by the UK blood services to obtain plasma, as far as possible, from male donors, to minimize the risk of TRALI caused by passive transfer of HLA or granulocyte antibodies arising in female donors as a result of pregnancy. The success of this policy has been measured by ongoing hemovigilance reporting; the number of cases of TRALI with high imputability reported to SHOT fell from 22 in 2003 to 13 in 2004, to six in 2005, and to three in 2006.<sup>44</sup>

Careful collection and analysis of hemovigilance data in the context of accurate denominator data has enabled risk estimation of transfusion in the developed world, as exemplified by Canada.<sup>53</sup> Such data must be made available, in comprehensible format, to doctors prescribing blood and patients receiving it, so that informed decisions can be made regarding risks and benefits, in the short and long term. Hemovigilance systems must be flexible, forward looking, responsive to developments in transfusion practice, and, above all, in touch with clinicians prescribing blood, and the requirements of patients.

## Disclaimer

The authors have disclosed no conflicts of interest.

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## Section IV: Part I

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### Appendix 44-1. ISBT Criteria for Severity and Imputability of Transfusion Reactions

| Grade/Score          | Definition                                                                                                                                                                                                                                                                                                                                            |
|----------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Severity</b>      |                                                                                                                                                                                                                                                                                                                                                       |
| 1 (non-severe)       | The recipient may have required medical intervention (eg, symptomatic treatment) but lack of such would not result in permanent damage or impairment of a body function.                                                                                                                                                                              |
| 2 (severe)           | The recipient required in-patient hospitalization or prolongation of hospitalization directly attributable to the event. OR<br>The adverse event resulted in persistent or significant disability or incapacity. OR<br>The adverse event necessitated medical or surgical intervention to preclude permanent damage or impairment of a body function. |
| 3 (life-threatening) | The recipient required major intervention following the transfusion (vasopressors, intubation, transfer to intensive care) to prevent death.                                                                                                                                                                                                          |
| 4 (death)            | The recipient died following an adverse transfusion reaction.*                                                                                                                                                                                                                                                                                        |
| <b>Imputability†</b> |                                                                                                                                                                                                                                                                                                                                                       |
| Definite (certain)   | When there is conclusive evidence beyond reasonable doubt that the adverse event can be attributed to the transfusion.                                                                                                                                                                                                                                |
| Probable (likely)    | When the evidence is clearly in favor of attributing the adverse event to the transfusion.                                                                                                                                                                                                                                                            |
| Possible             | When the evidence is indeterminate for attributing the adverse event to the transfusion or an alternate cause.                                                                                                                                                                                                                                        |
| Unlikely (doubtful)  | When the evidence is clearly in favor of attributing the adverse event to causes other than the transfusion.                                                                                                                                                                                                                                          |
| Excluded             | When there is conclusive evidence beyond reasonable doubt that the adverse event can be attributed to causes other than the transfusion.                                                                                                                                                                                                              |

\*Grade 4 should be used only if death is possibly, probably, or definitely related to transfusion. If the patient died of another cause, the severity of the reaction should be graded as 1, 2, or 3.

†Once the investigation of the adverse transfusion event has been completed, this is the assessment of the strength of relation to the transfusion of the event.

### Appendix 44-2. Standard for Collecting and Presenting of Data on Complications Related to Blood Donation

In order to facilitate international benchmarking, it is recommended that this standard be used when data on complications related to blood donation are presented. The Standard consists of

- Descriptions. A list with the categories grouped according to a code system.
- Data collection, Part 1. A list with description of the categories and definition of the grades of severity.
- Data collection, Part 2. An addendum with a form suitable for collecting data according to the standard.

The categories are divided in two main groups according to etiology and localization in the body. Some rare, but important, complications have been included in order to secure reports on occurrence. For the sake of simplicity they are grouped together under 300—Rare, important complications. The description of the categories includes definition, symptoms, complications, and grades of severity. Medical terms are defined as indicated in common medical dictionaries. The severity is graded according to duration of symptoms and of requirements for medical treatment.

The level of imputability is not included except for some of the complications in group 300—Rare, important complications.

If some of the symptoms essential for the definition of a category are missing, the complication can be registered in a more common or broad category. For example, if a donor complains of pain in the arm, the complication belongs to one of the following categories depending on when the symptoms occurred:

When needle was inserted Direct needle injury of a nerve (121).

After needle was inserted Indirect needle injury of a nerve by a haematoma (122).

However, if this information has not been registered the complication can be categorized as a nerve injury without specification (120).

## Complications Related to Blood Donation

### List of categories, numeric code, description, and grading (Version Madrid 2007)

| 100 Local Reactions Related to Needle Insertion |                                                          |                                                                                  |                                                                                                                                                                               |                                                                                                                                                                                 |
|-------------------------------------------------|----------------------------------------------------------|----------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Code                                            | Category                                                 | Definition                                                                       | Symptoms                                                                                                                                                                      | Grades                                                                                                                                                                          |
| <b>110</b>                                      | <b>Vessel injuries</b>                                   |                                                                                  |                                                                                                                                                                               |                                                                                                                                                                                 |
| 111                                             | Haematoma                                                | Collection of blood in the tissues                                               | Swelling, pain (local)                                                                                                                                                        | <b>Moderate</b> subjective symptoms<br><b>Severe</b> required medical treatment                                                                                                 |
| 112                                             | Arterial puncture                                        | Puncture of brachial artery                                                      | Swelling, pain (local)<br>Restricted movements of the elbow                                                                                                                   | <b>Moderate</b> observed (with or without symptoms)<br><b>Severe</b> required medical treatment                                                                                 |
| 113                                             | Thrombophlebitis                                         |                                                                                  | Swelling of the vein, pain (local)<br>Redness of the skin                                                                                                                     | <b>Severe</b> required medical treatment                                                                                                                                        |
| <b>120</b>                                      | <b>Nerve injuries</b>                                    |                                                                                  |                                                                                                                                                                               |                                                                                                                                                                                 |
| 121                                             | Injury of a nerve                                        | Direct nerve injury by the needle                                                | Severe, immediate pain when the needle was inserted which radiates down the forearm, often associated with paraesthesiae                                                      | <b>Mild</b> symptoms < 2 weeks<br><b>Moderate</b> symptoms > 2 weeks but < 1 year<br><b>Severe</b> symptoms > 1 year or required medical treatment                              |
| 122                                             | Injury of a nerve by a haematoma                         | Neurological symptoms caused by pressure from a haematoma                        | Pain and paraesthesia as 121 but symptoms develop some time after the needle was inserted                                                                                     | <b>Mild</b> symptoms < 2 weeks<br><b>Moderate</b> symptoms > 2 weeks but < 1 year<br><b>Severe</b> symptoms > 1 year or required medical treatment                              |
| <b>130</b>                                      | <b>Other complications (related to needle insertion)</b> |                                                                                  |                                                                                                                                                                               |                                                                                                                                                                                 |
| 131                                             | Tendon injury                                            | Direct tendon injury by needle                                                   | Severe, local pain when needle was inserted                                                                                                                                   | <b>Severe</b> required medical treatment                                                                                                                                        |
| 132                                             | Allergic reaction (local)                                |                                                                                  | At the venipuncture site – rash<br>swelling and itching                                                                                                                       | <b>Severe</b> required medical treatment                                                                                                                                        |
| 133                                             | Infection (local)                                        |                                                                                  | Swelling, redness and pain (local)                                                                                                                                            | <b>Severe</b> required medical treatment                                                                                                                                        |
| 200 General Reactions                           |                                                          |                                                                                  |                                                                                                                                                                               |                                                                                                                                                                                 |
| <b>210</b>                                      | <b>Vasovagal reactions</b>                               |                                                                                  |                                                                                                                                                                               |                                                                                                                                                                                 |
| 211                                             | Immediate type                                           | Symptoms occurred before donor left the donation site                            | Subjective symptoms: dizziness, nausea<br>Objective symptoms: Sweating, vomiting, pallor, hyperventilation, convulsions, loss of consciousness                                | <b>Mild:</b> Subjective symptoms only<br><b>Moderate:</b> Also objective symptoms<br><b>Severe:</b> Convulsions and/or Loss of consciousness and/or Required medical treatment  |
| 212                                             | Delayed type                                             | Symptoms occur after donor left the donation site but within 24 hours of leaving | Subjective symptoms: Discomfort, weakness, anxiety, dizziness, nausea<br>Objective symptoms: sweating, vomiting, pallor, hyperventilation, convulsions, loss of consciousness | <b>Mild:</b> Subjective symptoms only<br><b>Moderate:</b> Also objective symptoms.<br><b>Severe:</b> Convulsions and/or Loss of consciousness and/or Required medical treatment |

(Continued)

**List of categories, numeric code and description (Version Madrid 2007)**

| <b>300 Rare, Important Complications (The diagnosis must be confirmed by a medical doctor. Include only those complications which required medical treatment)</b> |                                                                                                    |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------|
| <b>310</b>                                                                                                                                                        | <b>Related to vessel injury</b>                                                                    |
| 311                                                                                                                                                               | <i>Brachial artery pseudoaneurysm</i>                                                              |
| 312                                                                                                                                                               | <i>Arteriovenous fistula</i>                                                                       |
| 313                                                                                                                                                               | <i>Compartment syndrome</i>                                                                        |
| 314                                                                                                                                                               | <i>Axillary vein thrombosis</i>                                                                    |
| <b>320</b>                                                                                                                                                        | <b>Accidents</b>                                                                                   |
| 321                                                                                                                                                               | Accidents related to vasovagal syncope (immediate and delayed)                                     |
| 322                                                                                                                                                               | Other accidents which occurred at the donation site                                                |
| <b>330</b>                                                                                                                                                        | <b>Cardiovascular reactions (symptoms commence within 24 hours of donor leaving donation site)</b> |
| 331                                                                                                                                                               | Angina pectoris                                                                                    |
| 332                                                                                                                                                               | Myocardial infarct                                                                                 |
| 333                                                                                                                                                               | Acute neurological condition (TIA, stroke)                                                         |
| <b>340</b>                                                                                                                                                        | <b>Related to apheresis procedures</b>                                                             |
| 341                                                                                                                                                               | Diffuse allergic reaction                                                                          |
| 342                                                                                                                                                               | Anaphylaxis                                                                                        |
| 343                                                                                                                                                               | Haemolysis                                                                                         |
| 344                                                                                                                                                               | Air embolus                                                                                        |
| <b>350</b>                                                                                                                                                        | <b>Death</b> – From any cause within 7 days of donation                                            |
| <b>360</b>                                                                                                                                                        | <b>Other</b>                                                                                       |

## Form that could be used for collection of data (Version Madrid 2007)

## DATA COLLECTION (part 1)

|            |  |                       |  |
|------------|--|-----------------------|--|
| Country:   |  | Period:               |  |
| Procedure: |  | Number of procedures: |  |

| Code                                           | Category                                                 | Severity                                       | Number |
|------------------------------------------------|----------------------------------------------------------|------------------------------------------------|--------|
| <b>100 Local Reactions to Needle Insertion</b> |                                                          |                                                |        |
| <b>110</b>                                     | <b>Vessel injuries</b>                                   |                                                |        |
| 111                                            | Haematoma                                                | moderate<br>severe<br><b>Sub total</b>         |        |
| 112                                            | Arterial puncture                                        | moderate<br>severe<br><b>Sub total</b>         |        |
| 113                                            | Thrombophlebitis                                         | severe                                         |        |
| <b>(111 + 112 + 113) Total</b>                 |                                                          |                                                |        |
| <b>120</b>                                     | <b>Nerve injuries</b>                                    |                                                |        |
| 121                                            | Injury of a nerve by the needle                          | mild<br>moderate<br>severe<br><b>Sub total</b> |        |
| 122                                            | Injury of a nerve by a haematoma                         | mild<br>moderate<br>severe<br><b>Sub total</b> |        |
| <b>(121 + 122) Total</b>                       |                                                          |                                                |        |
| <b>130</b>                                     | <b>Other complications (related to needle insertion)</b> |                                                |        |
| 131                                            | Tendon injury                                            | severe                                         |        |
| 132                                            | Allergic reaction (local)                                | severe                                         |        |
| 133                                            | Infection (local)                                        | severe                                         |        |
| <b>(131 + 132 + 133) Total</b>                 |                                                          |                                                |        |
| <b>200 General Reactions</b>                   |                                                          |                                                |        |
| <b>210</b>                                     | <b>Vasovagal reactions</b>                               |                                                |        |
| 211                                            | Immediate type                                           | mild<br>moderate<br>severe<br><b>Sub total</b> |        |
| 212                                            | Delayed type                                             | mild<br>moderate<br>severe<br><b>Sub total</b> |        |
| <b>(210 + 220) Total</b>                       |                                                          |                                                |        |

## Section IV: Part I

### DATA COLLECTION (part 2)

| Code                                     | Category                                   | Number |
|------------------------------------------|--------------------------------------------|--------|
| <b>300 Rare, Important Complications</b> |                                            |        |
| <b>310</b>                               | <b>Related to vessel injury</b>            |        |
| 311                                      | Brachial artery pseudoaneurysm             |        |
| 312                                      | Arteriovenous fistula                      |        |
| 313                                      | Compartment syndrome                       |        |
| 314                                      | Axillary vein thrombosis                   |        |
| (311+312+313+314+315) <b>Total</b>       |                                            |        |
| <b>320</b>                               | <b>Accidents</b>                           |        |
| 321                                      | Accidents related to vasovagal syncope     |        |
| 322                                      | Other kinds of accidents                   |        |
| (321+322) <b>Total</b>                   |                                            |        |
| <b>330</b>                               | <b>Cardiovascular reactions</b>            |        |
| 331                                      | Angina pectoris                            |        |
| 332                                      | Myocardial infarct                         |        |
| 333                                      | Acute neurological condition (TIA, stroke) |        |
| (331+332+333) <b>Total</b>               |                                            |        |
| <b>340</b>                               | <b>Related to apheresis procedures</b>     |        |
| 341                                      | Diffuse allergic reaction                  |        |
| 342                                      | Anaphylaxis                                |        |
| 343                                      | Haemolysis                                 |        |
| 344                                      | Air Embolus                                |        |
| (341+342+343+344) <b>Total</b>           |                                            |        |
| <b>350</b>                               | <b>Death</b>                               |        |
| <b>360</b>                               | <b>Other(s)</b>                            |        |

\*Developed jointly by a joint working group of the International Society of Blood Transfusion and the European Haemovigilance Network.

# Immunomodulatory and Proinflammatory Effects of Allogeneic Blood Transfusion

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As knowledge about the mechanisms of immune responsiveness and tolerance evolves, and as new tools to measure alterations in immunity become available, additional immunologic consequences of allogeneic blood transfusion (ABT) are detected. The lingering question has been whether these observations represent no more than laboratory curiosities, or whether they reflect some clinically relevant alteration in the recipient's immune function—the so-called “immunomodulatory” effect of ABT.<sup>1</sup> The constellation of all such ABT-associated laboratory and clinical findings is known as transfusion-related immunomodulation (TRIM). Initially, TRIM encompassed effects attributable to ABT by means of immunologic mechanisms only; however, more recently, the term has been used more broadly, to encompass additional effects that could be related to ABT by means of both immunomodulatory and proinflammatory rather than only immunomodulatory mechanisms.<sup>2</sup>

ABT may either cause alloimmunization or induce tolerance. ABT introduces a multitude of foreign antigens into the recipient, including HLA-DR antigens found on the donor's dendritic antigen-presenting cells (APCs). The presence or absence of autologous HLA-DR antigens on the donor's white blood cells (WBCs) plays a decisive role in whether alloimmunization or immune suppression will ensue following ABT.<sup>3</sup> Transfusions sharing at least one HLA-DR antigen with the recipient will induce tolerance, while fully HLA-DR-mismatched transfusions lead to alloimmunization.<sup>4</sup> In addition to the degree of HLA-DR compatibility between donor and recipient, the immunogenicity of cellular or soluble HLA antigens found in transfused blood components depends on the viability of the donor dendritic APCs and the presence of the required costimulatory signals for the presentation of the donor antigens to the recipient's T cells. Nonviable APCs and/or absence of the requisite costimulatory signals result in T-cell unresponsiveness.<sup>5–7</sup> Thus, when a multitude of antigens

is introduced into the host by ABT, the host response to some of these antigens is often decreased, and immune tolerance (or TRIM) ensues.<sup>8</sup> Several immune-function alterations have been documented in association with ABT (Table 45-1).<sup>9,10</sup>

All these ABT-related laboratory immune alterations could potentially be associated with clinical effects. Evidence from a variety of sources indicates that ABT enhances the survival of renal allografts<sup>11</sup>; may increase the recurrence rate of resected malignancies and the incidence of postoperative bacterial infections; may reduce the recurrence rate of Crohn's disease<sup>12</sup> and the risk of fetal loss in women with recurrent spontaneous abortions (RSAs); may activate infections with cytomegalovirus (CMV) or human immunodeficiency virus (HIV); and/or may increase short-term (up to 3 months after transfusion) mortality from all causes.<sup>13,14</sup>

Different biologic mechanisms may be involved in each of these purported clinical manifestations of TRIM, and the clinical evidence supporting each of the aforementioned hypotheses should be examined on its own merits.<sup>13,15</sup> The specific constituent(s) of allogeneic blood that mediate(s) the TRIM effect(s) also remain(s) unknown, and published literature has

**Table 45-1.** Documented Immune Function Alterations in Association with Allogeneic Blood Transfusion

- Decreased T-helper (CD4) cell count
- Decreased helper/suppressor (CD4/CD8) T-lymphocyte ratio
- Decreased lymphocyte response to mitogens
- Reduction in delayed-type hypersensitivity
- Decreased natural killer (NK) cell function
- B-cell activation
- T-cell activation
- Hypergammaglobulinemia
- Decreased cytokine (interleukin-2, interferon- $\gamma$ ) production
- Suppression of lymphocyte blastogenesis
- Decreased monocyte/macrophage phagocytic function
- Increased production of anti-idiotypic antibodies
- Increased production of anti-clonotypic antibodies

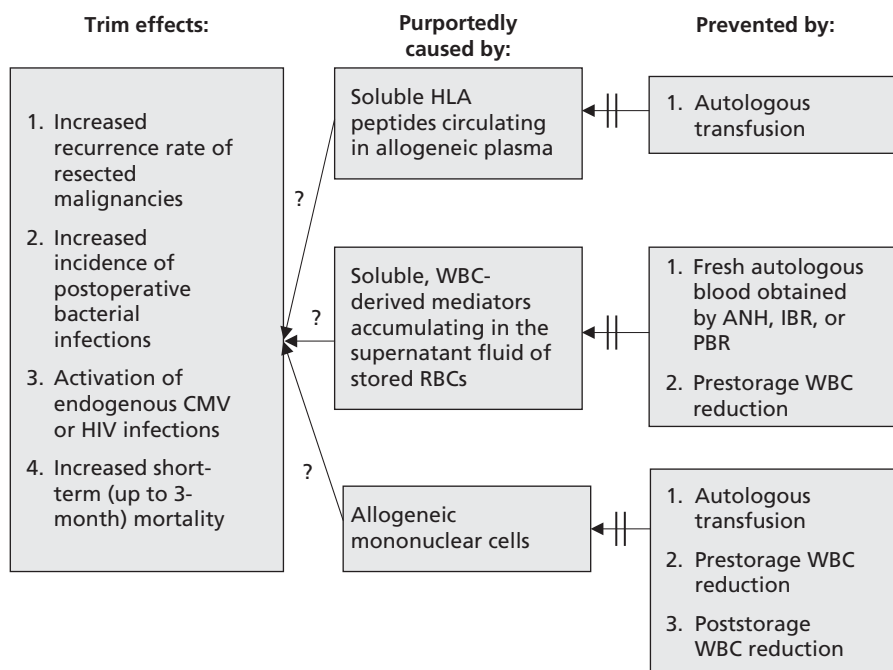


**Figure 45-1.** TRIM effects, postulated mediators of TRIM, and preventive strategies that could be effective if the TRIM effects were mediated by each corresponding mediator. The purported deleterious TRIM effects are mediated by an unknown constituent(s) of allogeneic blood that may (or may not) include one (or more) of the mediators shown. Stored autologous blood obtained by preoperative autologous blood donation is replete with white blood cell (WBC)-derived soluble mediators, because both autologous and allogeneic WBCs deteriorate equally during storage, releasing such mediators. Fresh autologous blood, transfused within hours of collection and processing, is free of WBC-derived soluble mediators and can be procured by acute normovolemic hemodilution (ANH), intraoperative blood recovery (IBR), or postoperative blood recovery (PBR). WBC-reduction filters do not retain WBC-derived soluble mediators, accounting for the difference between prestorage and poststorage WBC reduction in abrogating the TRIM effects mediated by such mediators. Modified with permission from Vamvakas.<sup>16</sup> CMV = cytomegalovirus; HIV = human immunodeficiency virus.

suggested that TRIM effects may be mediated by: 1) soluble HLA Class I peptides that circulate in allogeneic plasma; 2) soluble biologic response modifiers released in a time-dependent manner from WBC granules or membranes into the supernatant fluid of Red Blood Cell (RBC) or platelet concentrates during storage; and/or 3) allogeneic mononuclear cells (Fig 45-1).<sup>15</sup>

## Beneficial Clinical TRIM Effects

### Enhanced Survival of Renal Allografts

The only clearly established TRIM effect is beneficial—not deleterious: it is the enhanced survival of renal allografts in patients who have received pretransplant ABT.<sup>11,17</sup> Patients who receive allogeneic blood have been shown to have a significantly better renal allograft survival than untransfused patients, regardless of the number of HLA-A, HLA-B, and HLA-DR locus mismatches between recipient and donor.<sup>11,18</sup> This is true even when there is a common HLA haplotype, or shared HLA-B and HLA-DR antigens between donor and recipient.<sup>19</sup> The TRIM effect has also been reported with allografts between HLA-identical siblings.<sup>20</sup>

The ABT-related enhancement of renal allograft survival has been confirmed by animal data and clinical experience worldwide. In fact, it used to be standard policy in many renal transplant units to deliberately expose patients on transplant waiting lists to one or more allogeneic RBC transfusions. Subsequently, the beneficial effect of pretransplant ABT was thought to be less important—after cyclosporine and other potent immunosuppressive drugs were introduced. As a consequence, many centers discontinued its use. However, a multicenter observational study, reporting on 58,036 renal allografts from cadaveric donors after

the advent of cyclosporine, indicated that patients who received ABT were still more likely to have a successful renal allograft than those who did not.<sup>21</sup>

This study reported that the 1-year renal allograft survival of patients receiving pretransplant ABT was 3% to 5% better than that of those who did not receive ABT. Similar results were also reported for patients who received renal allografts from living-related donors.<sup>22</sup> The beneficial effect of pretransplant ABT in the outcome of cadaveric renal allografts was confirmed by a randomized controlled trial (RCT) conducted at 14 transplant centers.<sup>23</sup> Patients were randomly assigned to receive either three pretransplant, non-WBC-reduced RBC transfusions or no ABT. The renal allograft survival was significantly higher in the 205 transfused patients than in the 218 untransfused subjects (90% vs 82% at 1 year,  $p = 0.02$ ; 79% vs 70% at 5 years,  $p = 0.025$ ). The beneficial effect of ABT was found to be independent of age, gender, underlying disease, prophylaxis with lymphocyte antibodies, or the presence of preformed lymphocytotoxins.<sup>23</sup>

Two RCTs<sup>24,25</sup> have compared types of pretransplant ABTs given to prolong graft survival. Both studies were small, enrolling 52 and 144 patients, respectively. The first RCT<sup>24</sup> compared non-WBC-reduced and WBC-reduced RBCs and found no difference in graft survival. The other RCT<sup>25</sup> compared recipients of one HLA-DR-mismatched ABT, one HLA-DR-matched ABT, and no ABT. There was no difference in graft survival at 1 year or at 5 years.

Recipients of non-WBC-reduced whole blood or RBCs have better 1-year cadaveric allograft survival than patients given WBC-reduced blood components such as frozen-thawed-deglycerolized RBCs. These data indicate that allogeneic WBCs are involved in eliciting this beneficial TRIM effect.<sup>26</sup> However, the mechanism(s) involved in the ABT-related enhancement of renal allograft



survival remain(s) to be elucidated, and many questions about the optimal use of ABT in such patients remain to be answered. The latter include: the optimal number of ABTs, the volume of RBCs with each ABT, and the timing of the ABTs to produce the optimal clinical effect; and whether the TRIM effect of pretransplant ABTs poses any hazard(s) in addition to conferring benefit.

ABT administered during the actual operation for renal transplantation has not been shown to affect subsequent allograft survival.<sup>17</sup> Patients who receive more than 10 RBC units have a better 1-year allograft survival than patients who receive only 1 or 2 RBC units. However, patients who receive more than 10 RBC units appear to have a poorer overall allograft survival than those who receive fewer than 10 RBC units.<sup>27</sup> Such data suggest that multitransfused patients often develop cytotoxic antibodies and thus are at greater risk for earlier and more severe allograft rejection episodes.<sup>22</sup> Along these lines, Solheim<sup>28</sup> has reported another potential benefit from pretransplant ABTs, which is especially relevant in settings where there is a shortage of organs for transplantation. When several prospective recipients on a renal transplant list produce crossmatch-negative results with an available organ, pretransplant ABTs could help identify high-responder patients (ie, patients most likely to form cytotoxic antibodies in response to pretransplant ABTs, and also most likely to reject a transplanted kidney because of formation of cytotoxic antibodies after a negative crossmatch). Transplant surgeons could thus channel the scarce organ away from such patients and give it to a patient in whom it is most likely to survive.<sup>28</sup>

An experimental animal model has suggested that the beneficial effect of donor-specific ABT might be related to the type of transplanted organ. Whereas ABT appears to lead to permanent acceptance of all renal allografts, this benefit was not observed with pancreas, skin, or heart allografts.<sup>29</sup> Although additional data are required to understand how ABT induces its beneficial effect in renal transplantation, the authors believe that the use of pretransplant, non-WBC-reduced ABT remains a potentially useful intervention in the management of selected patients scheduled for renal transplantation even today; however, this benefit may not apply to settings where universal WBC reduction has been implemented.

### **Reduced Risk of Recurrent Spontaneous Abortions**

Transfusion of allogeneic WBCs has been proposed as a form of immunotherapy for the prevention of RSAs.<sup>30,31</sup> In this setting, the fetus represents a semi-allogeneic graft to its mother, and maintenance of a pregnancy depends on immunologic equilibrium between the implanted fetus and the maternal immune response to the fetus. When the genetic parents share HLA antigens, this balance may be altered and maternal blocking antibodies may not be formed, predisposing the pregnant woman to RSAs. Different transfusion protocols of allogeneic WBCs have been used at various centers to reduce the risk of RSAs, employing WBCs obtained from either sexual partners or third-party donors. Such WBCs have been administered intravenously, intracutaneously, or as an intradermal injection of mononuclear cells obtained by gradient separation. WBC-containing blood

components have included pooled buffy coats, single-donor buffy coats, or RBC suspensions containing WBCs.

The American Society for Reproductive Immunology (ASRI) conducted a worldwide collaborative individual-patient-data (IPD) meta-analysis to examine the efficacy of immunotherapy with allogeneic WBCs in patients with a history of RSAs<sup>32</sup> and concluded that such treatment was effective. The effect was small, with only 8% to 10% of affected women benefiting from the treatment. In contrast to the ASRI data, a recent multicenter RCT<sup>33</sup> that enrolled 183 women with a history of three or more spontaneous abortions reported a nonsignificant decrease in live-birth rate in patients randomly assigned to immunotherapy as compared with controls (36% vs 48%). However, the size of this RCT was likely inadequate to establish a clinically relevant treatment effect. Thus, the efficacy of transfusions of allogeneic WBCs for the treatment of patients with RSAs remains to be established.

### **Reduced Risk of Recurrence of Crohn's Disease**

Several observational studies have examined whether postoperative recurrence in patients with Crohn's disease—an immune-mediated disease—can be reduced by the perioperative administration of ABT. Pooled data from the available studies suggest that the recurrence rate in transfused and untransfused patients is similar: 37.5% vs 40.5%.<sup>12,34-37</sup> However, such integration of the reported data may be inappropriate, because the available studies are observational and medically heterogeneous (ie, using different follow-up periods and surgical interventions). Nonetheless, an IPD meta-analysis of 622 patients with Crohn's disease found no effect of perioperative ABT on subsequent need for surgical intervention, independent of age, gender, disease location, or extent of the resection.<sup>38</sup> Because many factors affect the risk of recurrence in patients with Crohn's disease, a large RCT is needed to clarify the role of ABT, if any, in modulating disease activity in patients with this disorder.

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## **Deleterious Clinical TRIM Effects**

### **Increased Recurrence of Resected Malignancies**

If ABT has a beneficial effect in renal transplantation, where immunosuppression is beneficial because it may prevent allograft rejection, ABT could also have deleterious effects in situations where impairment of the recipient's immune function can be detrimental. In 1981, 8 years after the first report of the beneficial effect of pretransplant ABT on renal allograft survival,<sup>11</sup> Gantt<sup>39</sup> voiced his concern that perioperative ABT for curative resection of a malignancy might provoke recurrence of cancer. Thus, if the host's immune response to a tumor contributes to controlling the tumor's growth, one would expect that impairment of the host's immunity, caused by ABT, would impair this defense mechanism and facilitate tumor growth.

More than 100 observational studies of ABT and cancer recurrence have been reported,<sup>40</sup> and their unadjusted results (ie, their findings before adjustment for the effects of confounding factors)

were subjected to three meta-analyses.<sup>40-42</sup> When the available results were integrated for seven cancer sites, there was agreement between the three overviews on the magnitude and statistical significance of the risk of cancer recurrence, death from cancer recurrence, or overall mortality in transfused compared with untransfused patients. A statistically significant adverse clinical outcome was found among transfused (compared with untransfused) patients for all cancer sites evaluated except for cervix.<sup>40</sup> A statistically significant ABT effect in the observational studies that had presented multivariate analyses adjusting for the effects of confounding factors was found in 24 studies, including 11 studies of colorectal cancer, four studies of head and neck cancer, one study of breast cancer, two studies of gastric cancer, four studies of lung cancer, and two studies of prostate cancer.<sup>40</sup> This ABT effect has not been confirmed by the available RCTs of perioperative ABT and cancer recurrence that are discussed later in this chapter (see TRIM effects mediated by allogeneic mononuclear cells).

### Increased Risk of Postoperative Bacterial Infection

None of the purported adverse TRIM effects of ABT has been established by RCTs, and the debate over the existence of clinical deleterious TRIM effects has been long and sometimes acrimonious.<sup>43-48</sup> Initially, the debate focused on differing interpretations of the findings of approximately 40 observational studies that had compared the risk of postoperative bacterial infection between transfused and untransfused patients undergoing gastrointestinal surgery, orthopedic operations, cardiac surgery, or various other surgical procedures. These studies tended to indicate that patients receiving perioperative ABT (compared with those not receiving ABT) almost always had a higher risk of developing postoperative bacterial infection.<sup>43</sup> The studies also indicated that patients receiving transfusion generally differed from those not receiving transfusion in several prognostic factors that predisposed to adverse clinical outcomes.<sup>44</sup> Based on these two sets of observations, some authors concluded that ABT has a direct deleterious effect on the recipient, causing an increased risk of postoperative bacterial infection.<sup>43</sup> Other investigators concluded that clinical need for ABT can be a surrogate marker for a variety of adverse prognostic factors and that the other variables that generated the need for ABT in the published studies also determined the subsequent clinical outcome.<sup>44</sup>

Today, the controversy over TRIM is focused on differing interpretations<sup>45-48</sup> of the findings of the available RCTs of perioperative ABT and postoperative infection. There have been 22 RCTs<sup>49-70</sup> that compared the risk of postoperative infection and/or mortality between patients randomly assigned to receive non-WBC-reduced allogeneic vs autologous or WBC-reduced allogeneic RBCs. Three of these RCTs<sup>50,52,71</sup> also compared the risk of cancer recurrence between the two randomization study arms. Nineteen RCTs were conducted in the perioperative setting; three more RCTs enrolled HIV-seropositive patients,<sup>62</sup> all hospitalized patients,<sup>65</sup> or trauma patients.<sup>70</sup>

Based on an integration of the results of all nine RCTs published or reported through 2002<sup>49,52,54,58-60,63,66,67</sup> and comparing the risk of postoperative infection between patients randomly

assigned to receive non-WBC-reduced vs WBC-reduced ABT in the event that they needed perioperative transfusion, two meta-analyses<sup>45,46</sup> concluded that non-WBC-reduced ABT is associated with postoperative infection. In contrast, a third meta-analysis<sup>47</sup> that integrated the findings of all 12 RCTs published or reported through 2005<sup>49,52,54,58-60,63,66-70</sup> found no association between non-WBC-reduced ABT and postoperative infection. Similarly, no association between ABT and postoperative infection was detected when the findings of RCTs<sup>50,51,55,56,64</sup> comparing recipients of allogeneic and autologous RBCs were integrated.<sup>72</sup>

The reasons for the disagreements between the three meta-analyses<sup>45-47</sup> that compared recipients of non-WBC-reduced vs WBC-reduced ABT have been discussed.<sup>48</sup> There were two main reasons for the disagreements: 1) the inclusion in the analysis of all 12 RCTs available today vs the nine initially published RCTs; and 2) the integration (or not) of the results of all 12 (or all nine) RCTs despite extreme medical heterogeneity. Medical heterogeneity refers to differences in study design with respect to such factors as the RBC product transfused to the non-WBC-reduced arm (ie, non-buffy-coat-reduced vs buffy-coat-reduced allogeneic RBCs or whole blood), the RBC product transfused to the WBC-reduced arm (ie, WBC-reduced RBCs or whole blood filtered before or after storage), the transfusion dose, the surgical setting (gastrointestinal, cardiac, or other), the types of postoperative infections evaluated, the criteria for diagnosing postoperative infection, and the frequency of postoperative infection recorded in each study.<sup>48</sup>

One would expect more of a TRIM effect in association with the transfusion of non-buffy-coat-reduced (compared with buffy-coat-reduced) allogeneic RBCs because the buffy-coat-reduced RBCs used in Europe contain only about one-third of the donor WBCs found in the non-buffy-coat-reduced RBCs used in North America. Second, one would expect a greater reduction of a TRIM effect(s) attributable to the transfusion of prestorage (compared with poststorage) filtered WBC-reduced allogeneic RBCs, because of the removal of WBC-derived soluble mediators through prestorage (but not poststorage) filtration (Fig 45-1). Third, one would expect more of a TRIM effect in cardiac (compared with other) surgical settings, because in cardiac surgery allogeneic mononuclear cells and/or WBC-derived soluble mediators might serve as a second inflammatory insult that compounds the diffuse inflammatory response to the extracorporeal circuit.<sup>73</sup>

Therefore, one would not expect all 12 RCTs available today (or all nine initially published RCTs) to have targeted the same TRIM effect. In contrast, depending on their design, these 12 (or nine) RCTs probably targeted TRIM effects that varied in magnitude and/or nature, making the integration of all 12 (or all nine) RCTs by a meta-analysis inappropriate. For example, RCTs administering RBCs filtered before storage to the WBC-reduced arm<sup>52,58,60,63,66-70</sup> investigated TRIM effects mediated by both WBC-derived soluble mediators and allogeneic mononuclear cells (Fig 45-1). However, RCTs transfusing RBCs filtered after storage to the WBC-reduced arm<sup>49,54,58,59</sup> investigated only those TRIM effects mediated by allogeneic mononuclear cells. Meta-analyses should integrate results only from subsets

of RCTs that are medically sufficiently homogeneous to justify the assumption that all combined studies have targeted a TRIM effect that is *biologically* similar.<sup>48</sup> Results from meta-analyses of such homogeneous subsets of RCTs are presented later in this chapter where TRIM effects mediated by WBC-derived soluble mediators or allogeneic mononuclear cells are discussed.

Blumberg et al<sup>46</sup> had earlier attributed the disagreements between the three meta-analyses<sup>45-47</sup> to the reliance on the intention-to-treat principle in the meta-analysis that did not detect an adverse TRIM effect of ABT<sup>47</sup> vs the use of results from “as-treated” analyses in the two meta-analyses that did detect a deleterious TRIM effect.<sup>45,46</sup> Intention-to-treat analyses often have reduced statistical power to detect a treatment effect compared with “as-treated” analyses.<sup>46</sup> However, both intention-to-treat and “as-treated” analyses demonstrated a deleterious TRIM effect when the analysis integrated the findings of *all* nine initially published RCTs. Neither the intention-to-treat nor the “as-treated” analysis showed an association between non-WBC-reduced ABT and postoperative infection when the analysis integrated the results of *all* 12 RCTs available today (Table 45-2).<sup>48</sup>

### Increased Risk of Short-Term Mortality

An association between non-WBC-reduced ABT and short-term (up to 3 months after transfusion) mortality from all causes was described in the RCT of van de Watering et al.<sup>58</sup> This RCT had been designed to investigate an association between non-WBC-reduced ABT and postoperative infection and, instead of that association, it observed an association between non-WBC-reduced ABT and mortality. The association between ABT and mortality was reported as a data-derived hypothesis,<sup>58</sup> and the authors postulated

that non-WBC-reduced ABT may predispose to multiple-organ failure (MOF), which might—in turn—predispose to mortality. These investigators undertook another RCT that confirmed the association between ABT and mortality but did not find an association between non-WBC-reduced ABT and increased MOF.<sup>63</sup>

Several preclinical<sup>74-78</sup> and clinical<sup>73,79-89</sup> observations have supported the hypothesis that ABT in general, and non-WBC-reduced ABT in particular, may be associated with MOF. The mechanisms underlying the development of MOF are unclear, but most evidence suggests that tissue injury is mediated by reactive oxygen species and proteolytic enzymes released from activated neutrophils.<sup>90-92</sup> Silliman et al<sup>74</sup> proposed that ABT may exercise a neutrophil-priming effect mediated by bioactive lipids that accumulate during storage. They postulated that rapidly deteriorating WBCs in stored RBC units release cytotoxic enzymes that may act on fragmented red cell membranes to produce mediators that are responsible for neutrophil priming and endothelial-cell activation (Fig 45-2).

These investigators<sup>74-76</sup> demonstrated that plasma obtained from stored RBC units primes neutrophils for superoxide production and enhanced cytotoxicity, and also activates pulmonary endothelial cells in a dose- and age-dependent fashion. The length of RBC storage was important in these studies, because no evidence of neutrophil priming was obtained when plasma stored for short periods was used. Silliman et al<sup>77</sup> also showed that lipids from the plasma supernatant of RBCs stored for 42 days cause acute lung injury in isolated pulmonary models. Similarly, Chin-Yee et al<sup>78</sup> reported that plasma supernatant from stored RBCs activates neutrophils. In that study, WBC reduction of the RBC units abrogated the effect.<sup>78</sup>

**Table 45-2.** Meta-Analyses of RCTs of Non-WBC-Reduced Allogeneic Blood Transfusion and Postoperative Infection: Impact of the Method of Analysis and the Number of RCTs Included in the Analysis<sup>48</sup>

| Number of RCTs included in the Analysis <sup>†</sup>                                          | Method of Analysis          |                    |                         |                                          |                    |                         |
|-----------------------------------------------------------------------------------------------|-----------------------------|--------------------|-------------------------|------------------------------------------|--------------------|-------------------------|
|                                                                                               | Intention-to-Treat*         |                    |                         | “As-Treated”*                            |                    |                         |
|                                                                                               | Number of Patients Analyzed | Summary Odds Ratio | 95% Confidence Interval | Number of Patients Analyzed <sup>‡</sup> | Summary Odds Ratio | 95% Confidence Interval |
| 9 RCTs <sup>49,52,54,58-60,63,66-67</sup> published or reported through 2002 <sup>45,46</sup> | 5017                        | 1.38               | 1.03-1.85 <sup>§</sup>  | 3265 (65.1%)                             | 1.56               | 1.06-2.31 <sup>§</sup>  |
| 12 RCTs <sup>49,52,54,58-60,63,66-70</sup> published or reported through 2005 <sup>47</sup>   | 6290                        | 1.24               | 0.98-1.56               | 4460 (70.1%)                             | 1.31               | 0.98-1.75               |

\*The intention-to-treat analyses included all patients randomly assigned preoperatively to receive non-WBC-reduced or WBC-reduced allogeneic blood in the event that they needed perioperative transfusion. The “as-treated” analyses retained only those patients from each randomization arm who ended up receiving transfusion during or after surgery.

<sup>†</sup>Integration of all nine (or all 12) RCTs, as shown in this table, is inappropriate because of the extreme medical heterogeneity of the studies (see text). Therefore, readers should resist the temptation to assign a medical or biological meaning to the figures presented in the table. Instead, readers are referred to Figs 45-4 and 45-5, which depict the results obtained when medically homogeneous subsets of these RCTs<sup>49,52,54,58-60,63,66-70</sup> were integrated.

<sup>‡</sup>The percentage of all randomized patients that was included in the “as-treated” analyses is given within parentheses.

<sup>§</sup>Statistically significant adverse ABT-related immunomodulatory effect ( $p < 0.05$ ).

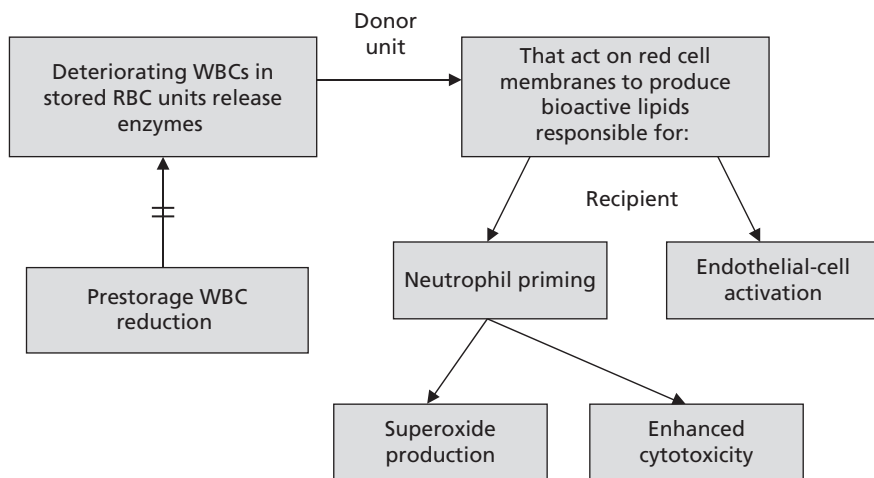


**Figure 45-2.** Proposed mechanism leading from the accumulation of bioactive lipids in the supernatant fluid of stored non-WBC-reduced Red Blood Cells (RBCs) to the development of multiple-organ failure in the recipient.<sup>74-78</sup>

Based on this observation that ascribes a neutrophil-priming effect to ABT (Fig 45-2), it is possible that the recently reported<sup>58,63</sup> association between ABT and short-term mortality could, in fact, reflect a “proinflammatory” rather than an “immunomodulatory” effect of ABT. However, for the time being and until these effects can be clearly defined, the authors believe that the association between ABT and MOF or mortality should be covered under the general overarching concept of adverse “TRIM” effects.<sup>2</sup>

In the study of Johnston et al,<sup>80</sup> patients receiving allogeneic RBCs had a significantly higher risk of MOF than recipients of polymerized hemoglobin. Neutrophils obtained from recipients of RBCs demonstrated priming, as evidenced by increased beta-2 integrin expression, superoxide production, and elastase release. Neutrophils obtained from recipients of polymerized hemoglobin showed no evidence of priming. Studies investigating the benefits obtained from placing a WBC reduction filter in the arterial line of the cardiopulmonary bypass circuit<sup>81-83</sup> suggested that non-WBC-reduced ABT may provoke cardiac and/or pulmonary failure. Furthermore, associations between ABT and prolonged mechanical ventilation<sup>84,85</sup> or MOF<sup>79,86-89</sup> were reported by some, but not all,<sup>93,94</sup> observational studies.

Recently, on the basis of an observational study of 248 patients, Netzer et al<sup>95</sup> reported that ABT in patients with acute lung injury was associated with increased in-hospital mortality. Following adjustment for confounding factors, ABT given after the onset of acute lung injury was associated with a 13% increase in mortality per unit of transfused RBCs ( $p < 0.0001$ ), but ABT given before the onset of acute lung injury was not associated with any higher risk. Both non-WBC-reduced and WBC-reduced ABT were associated with a significant ( $p < 0.001$ ) increase in mortality, although the magnitude of the increase in risk was greater when non-WBC-reduced (rather than WBC-reduced) RBCs had been used. Along the same lines, Murphy et al<sup>96</sup> linked the United Kingdom population register with the clinical, intensive-care-unit (ICU), hematology, and blood bank databases of 8516 patients who had undergone cardiac surgery over 8 years (1996-2003). When transfused and

untransfused patients were compared after adjustment for confounding factors, ABT was found to be associated with a higher risk of postoperative infection, ischemic postoperative morbidity, and early (0 to 30 days) as well as late (31 days to a year) mortality.

An association between non-WBC-reduced ABT and prolonged mechanical ventilation or MOF has not yet been reported by any RCT. Eleven RCTs, comparing recipients of non-WBC-reduced vs WBC-reduced allogeneic RBCs, and reporting on cancer recurrence, postoperative infection, or mortality as the primary outcome have presented information on short-term (up to 3 months after transfusion) mortality from all causes.<sup>54,57,58,60,63,65-70</sup> Because WBC reduction filters do not retain soluble mediators, if ABT exercised the described WBC-dependent neutrophil-priming effect mediated by bioactive lipids that accumulate during storage (Fig 45-2), allogeneic RBCs WBC-reduced before storage should abrogate this effect; but allogeneic RBCs WBC-reduced after storage should confer no benefit. Despite this theoretical prediction, no increase in mortality in association with non-WBC-reduced ABT was detected either across the subset of RCTs transfusing RBCs filtered before storage to the WBC-reduced arm, or across the subset of RCTs transfusing RBCs filtered after storage to the WBC-reduced arm.<sup>47</sup>

However, across five RCTs conducted in cardiac surgery<sup>58,63,66,68,69</sup> that had transfused RBCs filtered before storage to the non-WBC-reduced arm, non-WBC-reduced ABT was associated with a 72% increase in postoperative mortality [summary odds ratio, 1.72; 95% confidence interval (CI), 1.05–2.81;  $p < 0.05$ —Fig 45-3]. As already discussed, the TRIM effect seen across these studies may be associated with factors prevalent in the setting of patients undergoing cardiac surgery. For example, one can speculate that bioactive lipids accumulating during the storage of non-WBC-reduced RBCs and/or allogeneic mononuclear cells *may* represent a second inflammatory insult. That insult *may* compound the diffuse inflammatory response associated with the cardiopulmonary bypass circuit that *may* predispose recipients to MOF that *may*—in turn—predispose to mortality.<sup>73</sup>

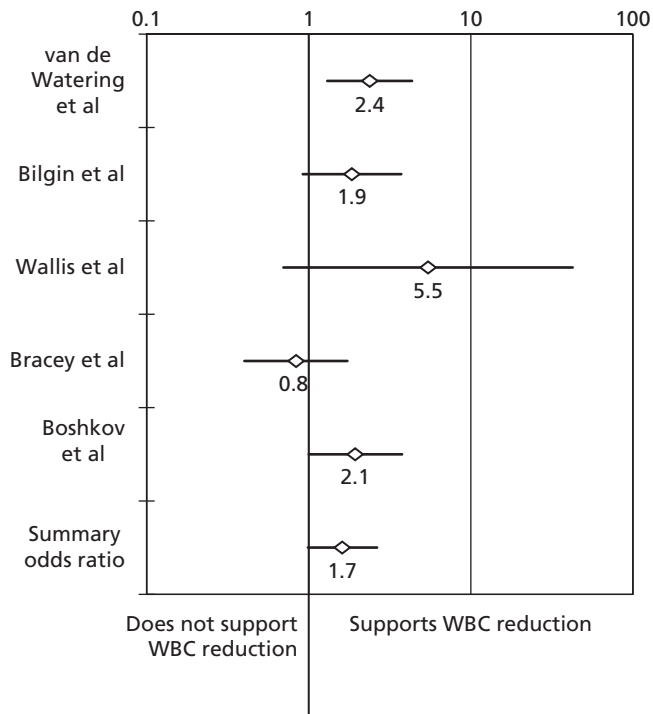


**Figure 45-3.** Postoperative mortality in cardiac-surgery patients. Randomized controlled trials (RCTs) investigating the association of non-WBC-reduced allogeneic blood transfusion (ABT) with short-term (up to 3 months after transfusion) mortality from all causes and conducted in the setting of cardiac surgery.<sup>58,63,66,68,69</sup> For each RCT, the figure shows the odds ratio (OR) of short-term mortality in recipients of non-WBC-reduced vs WBC-reduced allogeneic Red Blood Cells, as calculated from an intention-to-treat analysis; and the summary OR across the depicted RCTs, as calculated from a meta-analysis.<sup>47</sup> A deleterious ABT effect (and thus a benefit from WBC reduction) is demonstrated by an OR >1, provided that the effect is statistically significant ( $p < 0.05$ ; ie, provided that the associated 95% CI does not include the null value of 1).

### Effect of the Length of RBC Storage

If bioactive lipids or other soluble mediators accumulating in a time-dependent manner during storage were associated with adverse outcomes, observational studies should be reporting an association between prolonged storage of transfused non-WBC-reduced allogeneic RBCs and increased risk of occurrence of these adverse outcomes (because the longer the non-WBC-reduced allogeneic RBCs are stored, the higher the level of such soluble mediators they will contain). Also, the red cell storage lesion (Chapter 4) occurs in both the non-WBC-reduced and the WBC-reduced units, generating considerable numbers of nonfunctioning red cells. Such nonfunctioning red cells are removed from the recipient's circulation within 24 hours of the transfusion. Removal of large numbers of nonfunctioning red cells places a considerable burden on the reticuloendothelial system of a multitransfused recipient—a burden that could interfere with the host's response to bacteria and other challenges.

Nine observational studies have reported on the association between prolonged storage of transfused allogeneic RBCs and increased risk of morbidity or mortality. Four studies<sup>97-100</sup> reported on the association of prolonged storage of transfused RBCs with postoperative infection; one study<sup>79</sup> on the association between prolonged storage of transfused RBCs and MOF; and three studies<sup>101-103</sup> on the association between prolonged storage of transfused RBCs and increased mortality. The earlier studies<sup>79,97-102</sup> found prolonged storage of transfused non-WBC-reduced allogeneic RBCs to be associated with an increased risk of these adverse outcomes but van de Watering et al<sup>103</sup> found no association between prolonged storage time of transfused RBCs and increased mortality or longer stay in the ICU. Recently, in the setting of cardiac surgery, Gorman Koch et al<sup>104</sup> compared 2872 patients given RBCs stored for 14 days or less with 3130 patients given RBCs stored for more than 14 days. Prolonged storage was associated with an increase in the risk-adjusted rate of a composite of complications ( $p = 0.03$ ), while at 1 year mortality was significantly increased in patients given blood stored for more than 14 days (11.0% mortality) compared with patients given blood stored for 14 days or less (7.4% mortality). The effect of the length of storage on non-WBC-reduced RBCs on clinical outcomes has not yet been evaluated in RCTs.

### "Before-and-After" Studies

In the late 1990s, Canada and many western European countries implemented universal WBC reduction of cellular blood components by means of prestorage filtration. Once universal WBC reduction is implemented, the opportunity to conduct RCTs to establish adverse TRIM effects attributable to allogeneic WBCs is removed. It is, however, possible to compare the risk of infection or mortality in recipients of non-WBC-reduced RBCs before implementation of WBC reduction with the risk of infection or mortality in recipients of WBC-reduced RBCs after implementation of WBC reduction. Such "before-and-after" studies are observational, and—as such—they cannot establish causal relationships. Five before-and-after studies have reported data on the risk of infection,<sup>105-109</sup> and five such studies have reported data on the risk of short-term mortality.<sup>105,106,108-110</sup>

In Canada, Hébert et al<sup>108</sup> conducted a large study including 9525 patients undergoing cardiac surgery, 1731 patients undergoing orthopedic surgery, and 3530 patients admitted to the ICU. These investigators observed a statistically significant ( $p = 0.04$ ) decrease in short-term mortality (from 7.0% to 6.2%) after WBC reduction was introduced, without a concomitant reduction in the risk of postoperative infection. They offered the hypothesis that the observed decrease in the number of deaths might not have been mediated through suppression of the recipient's immune function, but through a proinflammatory microvascular effect of transfused WBCs that affects several organ systems. This hypothesis was buttressed by the findings of a companion before-and-after study in premature infants.<sup>109</sup> In that setting, the implementation of universal WBC reduction coincided with a reduction in several secondary morbidity outcomes from

several organ systems (ie, bronchopulmonary dysplasia, retinopathy of prematurity, necrotizing enterocolitis)—an observation consistent with a diffuse proinflammatory microvascular effect of allogeneic WBCs.

However, when all before-and-after studies were considered together in a meta-analysis,<sup>111</sup> and the findings of the unadjusted analyses from five studies<sup>105-109</sup> were integrated, there was an unadjusted association of WBC reduction with a decreased risk of postoperative infection. This association did not persist when findings from the multivariate analyses of the observational studies that had adjusted for the effects of confounding factors<sup>106,108,109</sup> were integrated. There was neither an unadjusted<sup>105,106,108-110</sup> nor an adjusted<sup>106,108,109</sup> association of WBC reduction with decreased short-term mortality.<sup>111</sup>

## TRIM Effects Mediated by Soluble Molecules Circulating in Allogeneic Plasma

### Soluble HLA Molecules

Soluble HLA proteins and immunoreactive HLA peptides remain important candidates as mediators of the TRIM effect(s). It has been suggested that nonpolymorphic peptides derived from HLA Class I molecules induce antigen-nonspecific immunosuppression, while polymorphic HLA Class I peptides have antigen-specific immunomodulatory effects.<sup>112-114</sup> It also seems possible that allogeneic plasma containing soluble HLA antigens may enter the recipient's thymic circulation, producing clonal deletion of the recipient's T cells that are directed against the allogeneic donor antigens.<sup>115,116</sup>

### Factor VIII Concentrates

In-vitro studies have indicated that low-molecular-weight components found in Factor VIII concentrates may inhibit the proliferative responses of peripheral blood mononuclear cells to phytohemagglutinin.<sup>117</sup> In these studies, high-purity Factor VIII concentrates have been shown to reduce the induction of T-cell-activation molecules such as the interleukin (IL)-2 receptor (CD25), the transferrin receptor (CD71), CD38, the CD11a/CD18 ratio, and HLA-DR antigen expression.<sup>117</sup> Moreover, evidence has been provided that this inhibitory action of Factor VIII concentrates was at least partly as a result of their contamination by transforming growth factor (TGF)- $\beta$ .<sup>118</sup>

### Autoantibodies

For some unknown reason, some people spontaneously produce large amounts of neutralizing autoantibodies to a number of growth factors (eg, granulocyte-macrophage colony-stimulating factor) or cytokines (eg, IL-1, IL-6, interferon- $\alpha$ ).<sup>119</sup> The autoantibodies in question are detectable in immunoglobulin preparations and correspondingly in plasma for transfusion. Therefore, if such donors donate blood, large amounts of neutralizing autoantibodies to growth factors or cytokines may be transferred to recipients by transfusion. In the plasma of transfusion recipients who had received plasma from donors with high titers of high-affinity neutralizing autoantibodies to IL-6 (0.1% of

normal donors), Hansen et al<sup>119</sup> demonstrated a 500-fold or greater increase in the concentration of complexed IL-6/autoantibody to IL-6, as compared with free IL-6 detectable in the patients' plasma before the transfusion.

When these autoantibodies reach a certain level, they may render a donor (or the recipient of plasma from such a donor) cytokine-deficient, but overt clinical sequelae of such a cytokine deficiency have not been reported. Hansen et al<sup>119</sup> christened this phenomenon transfusion-related inhibition of cytokines (TRICK). Depending on the cytokine or growth factor involved, TRICK could conceivably increase the transfusion recipient's susceptibility to infection or delay hematopoietic recovery after stem cell transplantation.

### Evidence from RCTs

The authors know of only one RCT<sup>66</sup> whose design permitted the investigators to examine the hypothesis that soluble plasma molecules circulating in allogeneic plasma may mediate TRIM effects. Wallis et al<sup>66</sup> randomly assigned 597 patients undergoing cardiac surgery to receive plasma-reduced, buffy-coat-reduced, or WBC-reduced allogeneic RBCs. In terms of their WBC content, plasma-reduced RBCs are equivalent to the buffy-coat-rich RBCs used in North America. The highest risk of postoperative infection was observed in the plasma-reduced study arm, in which the incidence of postoperative infection was 17.1%, as compared with 10.8% in the buffy-coat-reduced arm and 11.3% in the WBC-reduced arm ( $p = 0.20$ ). Although the difference between the three study arms was not significant, it appeared as though plasma removal did not confer a benefit with regard to the prevention of TRIM, or—by extension—that allogeneic plasma did not mediate TRIM.

## TRIM Effects Mediated by WBC-Derived Soluble Mediators

### Mediators Originating in WBC Granules

Biologic response modifiers accumulating in blood components during storage have been implicated in the pathogenesis of TRIM.<sup>120</sup> These mediators are contained in intracellular WBC granules, and are released in a time-dependent manner as the cells deteriorate.<sup>121</sup> Nielsen et al<sup>121</sup> reported that the concentration of histamine, eosinophil cationic protein, eosinophil protein X, myeloperoxidase, and plasminogen activator inhibitor-1 increase 3- to 25-fold in the supernatant fluid of RBC components between Days 0 and 35 of storage. Histamine, eosinophil cationic protein, and eosinophil protein X have been shown to inhibit neutrophil function, thereby contributing to the development of immunosuppression and tissue damage.<sup>122,123</sup>

### Soluble HLA Molecules and Fas Ligand

Soluble HLA molecules are present in the serum or plasma of healthy individuals. The liver is the main source of soluble HLA molecules found in the circulation. High levels of these molecules have been found in the serum or plasma of transplant recipients and patients with a variety of conditions, including

inflammatory, autoimmune, and infectious diseases. Soluble HLA molecules are also found in the supernatant fluid of stored RBCs and platelets, in direct proportion to the length of storage and the number of cells present. The biologic significance of these molecules has not been fully established, although it has been reported that they may be involved in the downregulation of the immune response and/or induction of tolerance.

Ghio et al<sup>124</sup> and Puppo et al<sup>125</sup> found soluble Fas-ligand (sFasL) and soluble HLA Class I molecules in the supernatant plasma of RBC and whole-blood-derived platelet units. The sFasL content of either 30-day stored RBCs or 5-day stored platelets was approximately 20 ng/mL. The infusion of sFasL in transfused blood components may bind the Fas molecule expressed on the natural killer (NK) and cytotoxic T cells of the recipient, thus preventing the binding of the Fas molecule on these immune cells to the Fas-ligand on virus-infected cells. Therefore, the infusion of sFasL in transfused blood components may impair the function of NK and cytotoxic T cells in the recipient, thus preventing apoptosis of virus-infected cells.<sup>126,127</sup> Ghio et al<sup>124</sup> and Puppo et al<sup>125</sup> demonstrated the functional capacity of sFasL molecules in stored blood components by culturing Jurkat cells in the presence of plasma supernatant from stored RBCs. Jurkat cells express Fas, and are thus susceptible to the effects of sFasL present in transfused blood components. In this in-vitro experiment, sFasL from the plasma supernatant of stored RBCs triggered apoptosis of the Jurkat cells, which was measured by flow cytometry.

These authors<sup>124,125</sup> also documented the accumulation of soluble HLA Class I molecules in stored RBCs and platelets, although the concentrations achieved were only 4 ng/mL in 30-day stored RBCs and 5-day stored platelets. Furthermore, stored supernatant plasma was shown to exercise an immunosuppressive effect in functional experiments, in that it inhibited the cytotoxic activity of lymphocytes known to be cytotoxic for cells infected with Epstein-Barr virus (EBV). This was not a nonspecific effect, because the cytotoxic activity of lymphocytes was restored after the stored supernatant plasma was depleted of soluble HLA Class I molecules. However, only supernatant plasma from stored non-WBC-reduced (as opposed to WBC-reduced) cellular blood components inhibited the cytotoxic activity of lymphocytes directed against EBV-infected cells. Similarly, prestorage WBC reduction prevented the accumulation of sFasL in stored RBCs.

### Apoptotic WBCs

Innerhofer et al<sup>128,129</sup> reported that impaired proliferative T-cell responses, decreased CD3+ counts, and a state of inappropriate immune activation, along with a diminished cytolytic response, occur even after transfusion of WBC-reduced RBCs containing a median residual WBC count of  $0.03 \times 10^6$  WBCs/unit (ie, a count far below the  $5 \times 10^6$  limit that qualifies a cellular blood component as “WBC-reduced”). If that were the case, it would appear that not only transfused intact, immunologically competent WBCs, but also transfused apoptotic or necrotic WBCs, could be important in provoking TRIM responses. Furthermore, activation of complement components<sup>130</sup> and formation of

anaphylatoxins<sup>131</sup> have also been reported during storage, but their significance in the context of TRIM is uncertain.

### Evidence from Animal Models

If soluble biologic response modifiers and remnants of apoptotic or necrotic WBCs accumulating in blood components during storage were shown to be responsible for some of the adverse TRIM effects of ABT, WBC reduction procedures intended to prevent such TRIM effects should be performed before storage, before WBC deterioration, and before the release of soluble biologic response modifiers from cell membranes or granules (Fig 45-1). The available WBC reduction filters do not retain soluble biologic response modifiers and are also ineffective in removing cell fragments. Therefore, both biologic response modifiers and the remnants of apoptotic or necrotic cells can be expected to persist in a blood component subjected to WBC filtration after storage. The importance of the timing of WBC reduction as regards the TRIM effect has been demonstrated in experimental animals.<sup>132</sup>

Bordin et al<sup>132</sup> showed that ABT promotes growth of established animal tumors and that the tumor-growth promoting effect of ABT can be ameliorated by prestorage (but not by poststorage) WBC reduction. These authors<sup>132</sup> used outbred New Zealand White (NZW) rabbits with established tumors as blood recipients, and outbred California Black rabbits as allogeneic blood donors. “Syngeneic” donor blood was collected from NZW rabbits who were littermates, or siblings, of the transfusion recipients. Non-WBC-reduced allogeneic, poststorage WBC-reduced allogeneic, prestorage WBC-reduced allogeneic, or syngeneic RBCs were transfused on Days 4 and 9 after the infusion of syngeneic epithelial tumor cells. All rabbits were killed 28 days after the infusion of the tumor cells, and the number of pulmonary tumor nodules was counted. Rabbits that received non-WBC-reduced allogeneic, poststorage WBC-reduced allogeneic, prestorage WBC-reduced allogeneic, or syngeneic RBCs had a median of 50.0, 39.0, 20.0, and 17.5 pulmonary nodules, respectively. The difference between non-WBC-reduced allogeneic and prestorage WBC-reduced allogeneic or syngeneic transfusion was highly significant ( $p < 0.0001$ ), but the difference between non-WBC-reduced allogeneic and poststorage WBC-reduced allogeneic transfusion was only marginally significant ( $p = 0.06$ ).

### Evidence from RCTs

With respect to infection, a recent theory<sup>133,134</sup> attributes the purported susceptibility of transfused patients to infection to a sustained inhibition of neutrophil chemotaxis caused by TGF- $\beta$ . TGF- $\beta$  renders neutrophils insensitive to chemotactic stimulation. Inhibition of chemotaxis is caused by both exogenous TGF- $\beta$ , contained in the supernatant of transfused blood components,<sup>133</sup> and endogenous TGF- $\beta$  produced by the recipient's neutrophils in response to sFasL and soluble HLA molecules found in the transfused supernatant.<sup>134</sup>

However, the results of meta-analyses of medically homogeneous subsets of RCTs that reported on postoperative infection

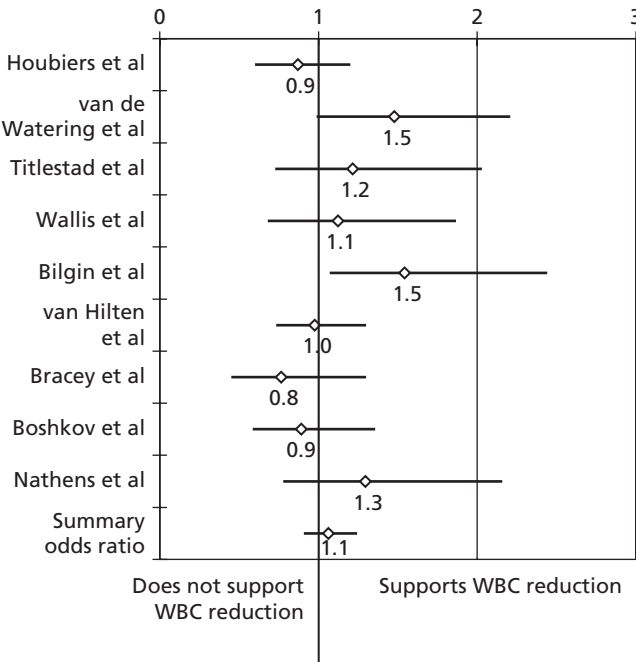


**Figure 45-4.** Risk of postoperative infection after transfusion of prestorage-filtered WBC-reduced RBCs. Randomized controlled trials (RCTs) of ABT and postoperative infection administering prestorage-filtered allogeneic RBCs to the WBC-reduced study arm.<sup>52,58,60,63,66-70</sup> The figure shows the odds ratio (OR) of postoperative infection in recipients of non-WBC-reduced vs WBC-reduced allogeneic RBCs, as calculated from an intention-to-treat analysis of each study; and the summary OR across the depicted RCTs, as calculated from a meta-analysis.<sup>47</sup> A deleterious ABT effect (and thus a benefit from WBC reduction) is demonstrated by an OR >1, provided that the effect is statistically significant ( $p < 0.05$ ; ie, provided that the associated 95% CI does not include the null value of 1).

contradicted the theory that attributes the TRIM effect to WBC-derived soluble mediators that accumulate during storage.<sup>47,48</sup> Across nine RCTs transfusing RBCs WBC-reduced before storage to the WBC-reduced study arm,<sup>52,58,60,63,66-70</sup> no TRIM effect was detected (summary odds ratio, 1.06; 95% CI, 0.91-1.24;  $p > 0.05$ ).<sup>47</sup> If the TRIM effect were mediated by WBC-derived soluble mediators, prestorage filtration should have abrogated an increased infection risk associated with non-WBC-reduced ABT, because it would have removed the allogeneic WBCs from the components given to the WBC-reduced arm of the studies before WBCs could release mediators into the supernatant fluid. Accordingly, a deleterious TRIM effect associated with non-WBC-reduced ABT would have been expected in this analysis, but the meta-analysis detected no such effect (Fig 45-4).

In contrast, across four RCTs<sup>49,54,58,59</sup> that transfused RBCs filtered after storage to the WBC-reduced arm, there was a more than twofold increase in the risk of infection in association with non-WBC-reduced ABT (summary odds ratio, 2.25; 95% CI, 1.12-4.25;  $p < 0.05$ —Fig 45-5). If the TRIM effect were mediated by WBC-derived soluble mediators, poststorage filtration should not have abrogated an increased infection risk associated with

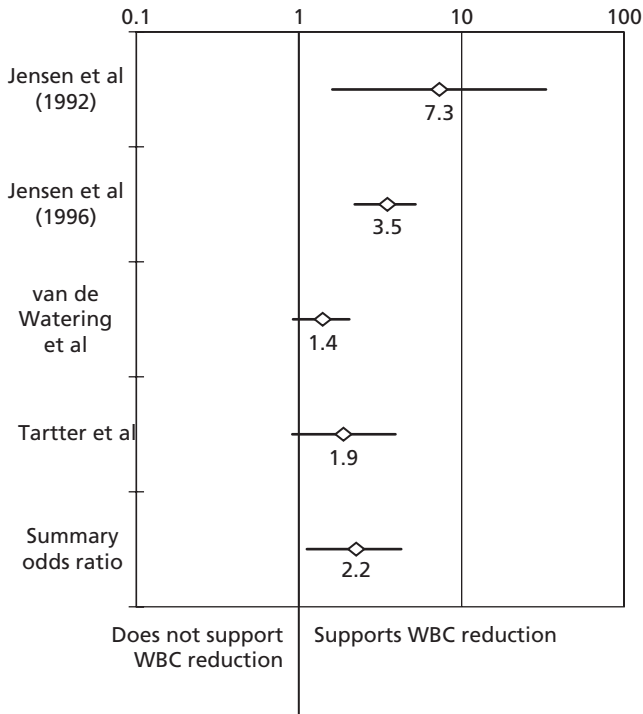


**Figure 45-5.** Risk of postoperative infection after transfusion of poststorage-filtered WBC-reduced RBCs or whole blood. Randomized controlled trials (RCTs) of ABT and postoperative infection administering poststorage-filtered allogeneic RBCs or whole blood to the WBC-reduced study arm.<sup>49,54,58,59</sup> The figure shows the odds ratio (OR) of postoperative infection in recipients of non-WBC-reduced vs WBC-reduced allogeneic RBCs or whole blood, as calculated from an intention-to-treat analysis of each study; and the summary OR across the depicted RCTs, as calculated from a meta-analysis.<sup>47</sup> A deleterious ABT effect (and thus a benefit from WBC reduction) is demonstrated by an OR >1, provided that the effect is statistically significant ( $p < 0.05$ ; ie, provided that the associated 95% CI does not include the null value of 1).

non-WBC-reduced ABT, because it would not have removed such mediators from the supernatant fluid of the stored RBCs given to the WBC-reduced arm of the studies. Thus, the large TRIM effect detected in this analysis (Fig 45-5) may be the result of the inclusion of three early RCTs<sup>49,54,59</sup> that had reported an unusually large TRIM effect.<sup>48</sup> These RCTs involved blood components no longer used in Western Europe or North America (allogeneic whole blood,<sup>49</sup> poststorage-filtered allogeneic whole blood,<sup>49</sup> or poststorage-filtered allogeneic RBCs<sup>54,59</sup>).

### TRIM Effects Mediated by Allogeneic Mononuclear Cells

The only established TRIM effect (ie, the beneficial effect of pre-transplant ABT on renal allograft survival) appears to require viable WBCs. Patients awaiting renal transplantation derive less immunologic benefit from pretransplant RBC transfusions that are WBC-reduced, washed, or frozen-thawed than from



non-WBC-reduced ABT. Mincheff et al<sup>135</sup> implicated the dendritic APCs of the allogeneic donor in the induction of a state of anergy in the recipient, proposing that during refrigeration APCs lose their ability to deliver costimulation. These investigators hypothesized that, following ABT, the recipient's T cells are stimulated by allogeneic donor APCs in the absence of costimulation, and this interaction induces a state of anergy in the recipient's T cells.

### Evidence from Animal Models

Animal data suggest that the TRIM effects are most likely mediated by transfused allogeneic mononuclear cells.<sup>136</sup> Kao<sup>137</sup> induced immune suppression in mice receiving donor WBCs free of plasma and platelets. A recent theory<sup>138</sup> proposes that donor dendritic cells expressing both alloantigen and the OX-2 (CD200) costimulatory molecule are required for the production of the TRIM effect.

CD200 is a transmembrane protein of the immunoglobulin superfamily that is expressed on various cell types, including a subpopulation of dendritic cells and some T and B cells.<sup>139</sup> Its receptor (CD200R or OX-2R) appears only on myeloid dendritic cells and some T cells. The interaction between CD200 and its receptor provokes a tolerance signal that leads to suppression of classical T-cell-mediated responses and the generation of  $\gamma\delta$ -suppressor T cells. Thus, CD200-deficient mice have reduced ability to downregulate activation of APCs. The absence of downregulatory signals in such mice results in exaggerated inflammatory responses and increased susceptibility to autoimmune encephalitis and collagen-induced arthritis. The interaction between CD200 and its receptor suppresses macrophage function, prolongs allograft survival, and prevents allogeneic fetal loss in a mouse model of cytokine-triggered abortions.<sup>140</sup>

Clark et al<sup>138</sup> demonstrated ABT-induced tumor growth in a murine model that employed BALB/c mice as allogeneic donors and C57Bl/6 mice as blood recipients. The recipient mice received a tail vein injection of syngeneic FSL10 fibrosarcoma cells, followed by transfusion of 50 to 200  $\mu$ L of allogeneic blood. Pulmonary tumor nodules were counted 3 weeks after the FSL10 cell infusion. There was a dose-response relationship between the volume of transfused allogeneic blood and the number of pulmonary tumor nodules, along with proliferation of TGF- $\beta$ -positive suppressor T cells in the spleen.

The tumor-growth-promoting effect of ABT was mediated by donor myeloid dendritic cells that expressed both CD11c and CD200 on their surface, because it could be blocked by monoclonal antibodies to either CD11c or CD200. (The effect could not be blocked by antibodies to CD200R, or by antibodies to other molecules participating in these interactions, an observation that implicated the subset of donor myeloid dendritic cells expressing both CD11c and CD200 in the pathogenesis of TRIM.) The interaction between the donor CD200 and its receptor on the recipient's T cells induced proliferation of  $\gamma\delta$ -suppressor T cells that released cytokines, especially TGF- $\beta$ . Physiologic concentrations of TGF- $\beta$  stimulated proliferation of FSL10 fibrosarcoma cells in vitro. Because TGF- $\beta$  can also suppress host

defenses against infectious agents,<sup>141</sup> it could be the basis of the TRIM effect with regard to both postoperative infections and tumor growth, at least as regards sarcomas.

Bordin and Blajchman<sup>142</sup> reviewed the findings of animal models of ABT and cancer recurrence and reported that 17 published models had found stimulation of tumor growth by ABT, as compared with three models that had reported inhibition of tumor growth and four models that had found no effect. Data from both inbred and outbred animal models have indicated that ABT accelerates tumor growth and enhances formation of metastatic nodules.<sup>132,143-146</sup> Allogeneically transfused mice inoculated intramuscularly with either syngeneic malignant melanoma (B16) or mastocytoma (P815) cells developed larger tumors than did syngeneically transfused mice.<sup>143</sup> Similar results were obtained when syngeneic B16 tumor cells were infused intravenously and the numbers of pulmonary nodules enumerated.<sup>143,144</sup> Experiments performed to investigate the effect of the tumor-cell dose showed that the ABT effect was only evident when small numbers ( $1.25\text{--}2.5 \times 10^5$ ) of tumor cells were inoculated into the host animal. The effect was not evident when large numbers of tumor cells were inoculated, suggesting that the tumor burden had a strong bearing on whether the ABT effect became manifest.

The influence of the timing of ABT in enhancing pulmonary-nodule formation has also been examined. Studies in both inbred (mice) and outbred (rabbits) animals have shown that ABT has a tumor-growth-promoting effect when administered before the infusion of syngeneic tumor cells.<sup>132,145</sup> In the murine model, male C57Bl/6J mice (MHC type H-2b) were blood recipients; Balb/c mice (MHC type H-2d) were allogeneic donors; and the tumor cells were syngeneic (H-2b) methylcholanthrene-induced fibrosarcoma cells.<sup>145</sup> As already discussed, to better replicate the situation seen clinically, the enhancement of tumor growth by ABT has been investigated in animals (mice and rabbits) that received such syngeneic and allogeneic transfusions subsequent to the inoculation of the tumor cells, and the data indicated that ABT enhanced tumor growth also in animals with established tumors.<sup>132</sup> Using inbred animals (mice) only, another series of investigations provided similar evidence indicating that ABT given after tumor-cell engraftment enhanced tumor growth.<sup>146</sup> It was also shown that animals with either nonestablished or established tumors receiving non-WBC-reduced ABT developed significantly larger numbers of pulmonary nodules than did animals given WBC-reduced ABT.<sup>132,145</sup>

Finally, the tumor-growth-promoting effect of ABT can be adoptively transferred to naive animals by spleen cells harvested from allogeneically transfused animals.<sup>145</sup> In these experiments, the number of pulmonary nodules observed in animals that had received spleen cells from allogeneically transfused animals was significantly higher than that observed in animals that had received spleen cells from animals transfused with syngeneic blood. However, the ABT effect could not be adoptively transferred to naive animals that received spleen cells derived from animals transfused with prestorage-WBC-reduced allogeneic blood.

The clonal deletion seen in recipients of ABT refers to the removal of lymphocytes that promote the clearance of transfused alloantigens. Interactions between Fas and FasL are involved in the clonal deletion of T cells and the downregulation of cytotoxic T-cell activity. In a murine model, Hashimoto et al<sup>147</sup> investigated the possibility of splenic-lymphocyte deletion secondary to ABT-related augmentation of apoptosis. These investigators demonstrated that non-WBC-reduced ABT upregulated the expression of Fas and FasL on CD4+ as well as CD8+ splenic T cells and could thereby promote their apoptosis. The ABT-related immune alterations could be partially prevented by WBC reduction of the transfused blood, as CD8+ splenic cells from mice receiving non-WBC-reduced ABT showed higher expression of Fas and FasL than cells from mice receiving WBC-reduced ABT.

The data regarding the TRIM effect and infection in animal models are contradictory. Moreover, a variety of experimental conditions such as anesthesia, shock, trauma, type of surgery, blood volume, as well as timing and transfusion frequency have all been reported to have an impact on the results.<sup>148-152</sup> In a series of studies in experimental animal, Waymack et al<sup>148-151</sup> have demonstrated that allogeneically transfused animals had immune impairment and a poorer response to a septic challenge than did syngeneically transfused animals. In a burn model, these investigators observed that rats given ABT had higher mortality than did rats given syngeneic blood or saline.<sup>152</sup> In a rat bacterial-peritonitis model, a significant adverse effect on survival was associated with ABT.<sup>149</sup> In another study, ABT was associated with marked immune impairment to a bacterial challenge immediately after the transfusion.<sup>153</sup>

Moreover, ABT and, in particular, transfused allogeneic WBCs adversely affected host resistance to a gut-derived infection with *Escherichia coli* in a murine model.<sup>154</sup> In addition, in a cecal ligation and puncture murine model, ABT greatly increased susceptibility to infection. These studies also indicated that spleen cells of allogeneically transfused mice produced increased quantities of the Th-2 cytokines (IL-4 and IL-10 and lesser amounts of IL-2), probably leading to increased antibody production and a decreased cell-mediated response.<sup>155</sup> In contrast, in murine experiments using a bacterial-peritonitis model that compared syngeneic with allogeneic transfusion, the latter was shown not to influence overall survival of animals challenged with *E. coli*.<sup>156</sup> Similarly, while a clear negative effect of shock was detected, no adverse effect of transfusions, either syngeneic or allogeneic, was observed in a rat model.<sup>157</sup>

### Microchimerism

HLA compatibility between donor and recipient may result in the persistence of allogeneic donor WBCs, including dendritic APCs, in the recipient. Such long-term engraftment and survival of small numbers of donor cells (microchimerism) has been proposed as a possible mechanism of TRIM.<sup>158</sup> Microchimerism could cause the downregulation of the recipient's immune response, resulting in tolerance to donor alloantigens and allograft survival. Microchimerism results in the release of IL-4,

IL-10, and TGF- $\beta$  from T-helper type 2 (Th-2) lymphocytes.<sup>159</sup> These cytokines have been shown to inhibit the production of T-helper type 1 (Th-1) cells and to deactivate cytotoxic T cells, thereby suppressing allograft rejection. Along similar lines, ABT was shown to cause a shift in peripheral T-cell cytokine secretion toward that of the Th-2 phenotype, and to downregulate Th-1 cytokine secretion. Impairment of Th-1 cytokine secretion results in impairment of various functions of cellular immunity (including antigen processing, macrophage activation, the T-cell cytotoxic function, and the neutrophil and monocyte cytotoxic activity) that are supported by Th-1 cytokines, such as IL-2, IL-12, and interferon- $\gamma$ .<sup>160</sup>

In 1995, Lee et al<sup>161</sup> employed quantitative allele-specific polymerase chain reaction methods to demonstrate a thousand-fold expansion of allogeneic donor WBCs in the recipient's circulation 3 to 5 days following transfusion in otherwise healthy adults undergoing elective orthopedic surgery. The allogeneic WBCs were cleared from the circulation within 2 weeks. The finding was verified in a canine transfusion model and—as expected—irradiation of blood products abrogated the allogeneic donor WBC expansion phase. However, in 1999, the same group documented high-level and long-lasting WBC microchimerism among selected victims of traumatic injury who had received a large number of very fresh units of blood during resuscitation.<sup>162</sup> In some of these trauma patients, up to 3% to 4% of circulating WBCs were of donor origin as long as 2 years following transfusion. Analysis of lymphocyte subsets using immunomagnetic bead enrichment showed that both lymphoid and myeloid lineages were represented.

Long-term transfusion-associated microchimerism appears to be a common, albeit only recently recognized, complication of ABT<sup>163</sup> that has hitherto been demonstrated only in trauma patients.<sup>162</sup> Injury produces an immunosuppressive and inflammatory milieu in which very fresh blood components, containing WBCs capable of replication, are often transfused in large quantities. Transfusion-associated microchimerism is present in approximately half of transfused, severely injured patients at hospital discharge.<sup>163</sup> In approximately 10% of the patients, the chimerism associated with a single blood donor may increase in magnitude over months to years, representing up to 2% to 5% of circulating WBCs.<sup>162,163</sup> Nonetheless, in other patient populations, such as those infected with HIV, ABT-induced microchimerism appears to be transient.<sup>164</sup>

If microchimerism does lead to the downregulation of the host's immune response, it is important to note that microchimerism is detected also following administration of WBC-reduced allogeneic RBCs.<sup>165</sup> Utter et al<sup>166</sup> examined a subgroup of the trauma patients enrolled in the RCT of Nathens et al<sup>70</sup> that had randomly assigned patients to receive non-WBC-reduced or WBC-reduced allogeneic RBCs filtered before storage. Nine of 32 (28%) patients in the non-WBC-reduced group developed microchimerism, as compared with 13 of 35 (37%) patients in the WBC-reduced group ( $p = 0.43$ ).<sup>166</sup> Several months after the transfusion, subjects with transfusion-associated

microchimerism were no more likely than subjects without transfusion-associated microchimerism to have at least one symptom suggestive of chronic graft-vs-host disease (64% vs 76%, respectively), indicating that transfusion-associated microchimerism is prevalent in this patient population but unlikely to be associated with symptoms.<sup>166</sup>

Similarly, Fresland et al<sup>167</sup> reported that microchimerism after ABT could be induced by transfusion of RBCs that were WBC-reduced by prestorage filtration. Moreover, in that study,<sup>167</sup> microchimerism was not dose dependent, and it could be induced even by RBC units that had been stored for more than 3 weeks.

### Evidence from RCTs

The authors know of only one RCT<sup>62</sup> that had been specifically designed to test a possible TRIM effect of allogeneic mononuclear cells. The Viral Activation Transfusion Study (VATS)<sup>62</sup> transfused unmodified allogeneic RBCs stored for <2 weeks (and thus containing relatively undamaged allogeneic mononuclear cells) to the non-WBC-reduced arm. Also, the two study arms were controlled for comparable duration of storage of the transfused RBCs. There was no difference between the study arms in the HIV or CMV viral load or the length of survival. Median survival was 13.0 months in recipients of prestorage-filtered allogeneic RBCs, as compared with 20.5 months in recipients of non-buffy-coat-reduced allogeneic RBCs ( $p = 0.12$ ). Thus, the VATS results have impugned the theory attributing the TRIM effect to allogeneic mononuclear cells. It should be noted, however, that no other RCT transfusing fresh components to the non-WBC-reduced study arm has been reported, and the effect of fresh components has not been studied in the context of more “traditional” TRIM effects (ie, cancer recurrence or postoperative infection).

Despite the convincing evidence provided by animal models for a relationship between transfusion of allogeneic mononuclear cells and tumor recurrence, no RCT of ABT and cancer recurrence has transfused fresh non-WBC-reduced RBCs to the non-WBC-reduced study arm to test for the effect of immunologically competent allogeneic mononuclear cells seen in animal models. Moreover, no RCT of ABT and cancer recurrence has enrolled patients with sarcomas—tumors whose growth is stimulated by TGF- $\beta$ <sup>138</sup>—or patients with tumors for which the immune response plays a major role. (These include skin tumors—such as melanomas, keratoacanthomas, squamous and basal-cell carcinomas—and certain virus-induced tumors—notably Kaposi’s sarcoma and certain lymphomas.<sup>168</sup>) Instead, the three available RCTs<sup>50,52,71</sup> of ABT and cancer recurrence enrolled patients with colorectal cancer—a tumor that is not sufficiently antigenic to render an impairment of the host’s immunity capable of facilitating tumor growth, and whose cells have not been shown to be stimulated by TGF- $\beta$ .

Thus, these three RCTs<sup>50,52,71</sup> permit very limited inference with regard to the biologic significance of the TRIM mediators discussed in this chapter. No adverse TRIM effect of ABT on cancer recurrence is detected across the three

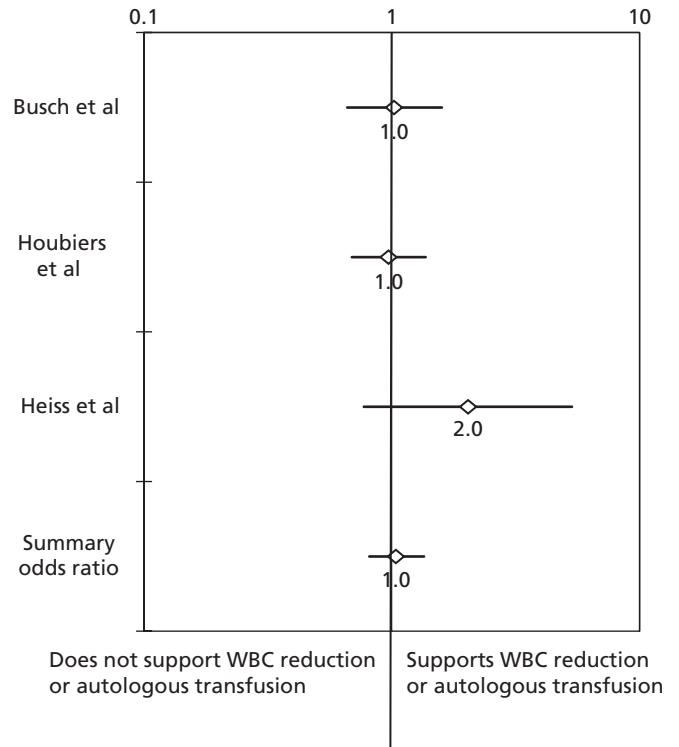


**Figure 45-6.** Cancer recurrence after transfusion. Randomized controlled trials (RCTs) investigating the association of non-WBC-reduced allogeneic blood transfusion (ABT) with cancer recurrence.<sup>50,52,71</sup> For each RCT, the figure shows the odds ratio (OR) of cancer recurrence in recipients of non-WBC-reduced vs WBC-reduced allogeneic or autologous RBCs or whole blood, as calculated from an intention-to-treat analysis; and the summary OR across the depicted RCTs, as calculated from a meta-analysis.<sup>167</sup> Each OR is surrounded by its 95% confidence interval (CI). A deleterious effect of ABT (and thus a benefit from autologous transfusion or WBC reduction) is demonstrated by an OR > 1, provided that the effect is statistically significant ( $p < 0.05$ ; ie, provided that the associated 95% CI does not include the null value of 1).

studies (Fig 45-6). The summary odds ratio of cancer recurrence in recipients of non-WBC-reduced allogeneic compared with autologous or WBC-reduced allogeneic RBCs is 1.04 (95% CI, 0.81-1.35;  $p > 0.05$ ).<sup>169</sup>

### Summary and Conclusions

TRIM encompasses the laboratory immune aberrations that occur after ABT (Table 45-1) and their established or purported clinical effects. TRIM is a real biologic phenomenon resulting in at least one established beneficial clinical effect in humans (the enhanced survival of renal allografts), but the existence of deleterious clinical TRIM effects has not yet been confirmed. Initially, TRIM encompassed effects attributable to ABT by immunomodulatory mechanisms (eg, cancer recurrence, postoperative infection, or virus activation); more recently, TRIM has also included effects attributable to ABT by proinflammatory mechanisms (eg, multiple-organ failure or mortality).

The mechanism(s) of the TRIM effect(s) remain(s) elusive, and it is possible that a large number of biologic mechanisms may underlie these effect(s). The infusion of foreign antigen in either soluble or cell-associated form has been shown to induce immune suppression and anergy, as well as clonal deletion in studies in experimental animals. However, most studies evaluating proposed mechanisms have been conducted in rodents, and the findings may not be applicable to the human immune system.<sup>170</sup> Dzik<sup>171</sup> suggested that there may be two categories of a TRIM effect: one that is HLA-dependent and directed against adaptive immunity and another that is nonspecific and directed against innate immunity. The nonspecific effect might result from the infusion of WBCs that undergo apoptosis during refrigerated storage. Immunosuppression resulting from the infusion of apoptotic cells may be linked to TGF- $\beta$ .

Support for the theory that TRIM is caused by the allogeneic WBCs has come mainly from data from animal models. These have shown that animals receiving allogeneic buffy-coat leukocytes develop significantly more pulmonary tumor nodules than do animals given either plasma or prestorage-WBC-reduced whole blood.<sup>132</sup> It is possible that prestorage WBC reduction may prevent the accumulation of soluble mediators that are actively synthesized and released by WBCs during RBC storage, and that such WBC-derived soluble mediators are involved in the immunomodulation observed following ABT. However, storage lesions of RBCs and platelets occur even when the units have been WBC-reduced before storage (WBC removal only slightly improves these storage lesions—see Chapters 4 and 12).

The totality of the evidence from RCTs does not demonstrate the kind of deleterious TRIM effect that would justify universal WBC reduction specifically for prevention of this effect (ie, a TRIM effect manifest across all clinical settings and transfused RBC products), although universal WBC reduction may be justified on the basis of other WBC-related adverse effects.<sup>136</sup> Non-WBC-reduced ABT is associated with an increased risk of short-term (up to 3 months after transfusion) mortality from all causes specifically in cardiac surgery. Even in this setting, the reasons for the excess deaths attributed to non-WBC-reduced ABT remain elusive. The initial hypothesis suggested that non-WBC-reduced ABT may predispose to MOF which, in turn, may predispose to mortality.<sup>58</sup> However, no cardiac-surgery RCT has demonstrated an association between non-WBC-reduced ABT and MOF.

The TRIM effect seen in cardiac surgery deserves further study to pinpoint the cause(s) of the excess deaths, but—now that the majority of transfusions in Western Europe and North America are WBC-reduced—the undertaking of further RCTs comparing recipients of non-WBC-reduced vs WBC-reduced RBCs in cardiac surgery is difficult. Until further studies are conducted, whether to opt for WBC reduction of all cellular blood components transfused in cardiac surgery (in the absence of information on the specific cause or causes of death that can be ascribed to non-WBC-reduced ABT) is a policy decision that will have to be made on the basis of available data (Fig 45-3). The authors believe that WBC reduction of all cellular blood

components transfused in cardiac surgery is appropriate based on the accumulated evidence.

It must be emphasized that, at least in the case of some adverse TRIM effects, the evidence for the existence of such effects may not be available because the requisite studies have not been conducted. An effect of the transfusion of allogeneic mononuclear cells would be expected to occur on tumor recurrence for example, based on the convincing findings from experimental animals. However, no available RCT has transfused fresh non-WBC-reduced RBCs to the non-WBC-reduced study arm to specifically study the effect of allogeneic mononuclear cells. Moreover, no available RCT has enrolled patients with a tumor whose growth would be expected to be stimulated by ABT. A possible adverse TRIM effect of allogeneic mononuclear cells has similarly not been adequately investigated in the areas of postoperative infection and mortality.

Indeed, in many cases, the preclinical studies were conducted and the hypotheses about mechanisms formulated after clinical studies (including RCTs) had already presented data-derived hypotheses to account for unexpected ABT effects. Because it has not been possible to conduct further RCTs after the hypotheses about TRIM mediators (Fig 45-1) were crystallized, whether some adverse TRIM effects exist (or not) in humans may remain a mystery. Moreover, it is possible that the available RCTs have targeted outcomes that did not capture the true nature of the ABT effect. If this effect were “proinflammatory” rather than “immunomodulatory,” it would have been expected to result not in clinical impairment of the recipient’s immunity, but in multiple-organ dysfunction. MOF and related outcomes were not studied in most completed RCTs.

New data continue to appear,<sup>172-177</sup> and the controversy on the purported deleterious TRIM effects continues to persist.<sup>45-48,178-182</sup> Because the use of WBC-reduced allogeneic RBCs and platelets is becoming widespread, the opportunity to conduct RCTs to establish the existence of adverse TRIM effects mediated by allogeneic WBCs is removed. Despite the widespread acceptance of WBC reduction as a standard of care in many countries, however, it remains unclear whether deleterious clinical TRIM effects of ABT truly exist, and—in the event that they do—whether they are mediated, directly or indirectly, by allogeneic WBCs. The uncertainties and hypotheses discussed here underline the need for further research!

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## Disclaimer

The authors have disclosed no conflicts of interest.

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## Transfusion-Transmitted Hepatitis

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Hepatitis B virus (HBV) infection, originally called serum hepatitis, was definitively shown to be a serious, and sometimes fatal complication of blood transfusion when unscreened blood and plasma were massively transfused during World War II. Despite this clinical recognition, little was known of the causative agent until the 1960s when the serendipitous finding of an antigen in the serum of an Australian aborigine (Australia antigen)<sup>1</sup> ultimately led to the linkage of this antigen to human hepatitis.<sup>2</sup> Further investigation revealed that the Australia antigen represented the surface protein of a viral particle (Dane particle) that was later shown to be the hepatitis B virion. Once this relationship was understood, the Australia antigen was renamed the hepatitis B surface antigen (HBsAg) and tests to detect this antigen in blood donors were introduced in the United States in 1970. This screening measure, plus the universal adoption of an all volunteer donor system, resulted in a near 70% reduction in total posttransfusion hepatitis and a greater than 85% reduction in transfusion-associated hepatitis B.<sup>3</sup> Improvements in test sensitivity for HBsAg and the addition of screening assays for the detection of antibody to the hepatitis B core (nucleocapsid) protein (anti-HBc) in 1987 further reduced transfusion-associated hepatitis B to a calculated incidence of approximately one infection in every 150,000 transfusions. Rarely do these infections result in clinical disease and  $\geq 95\%$  of HBV infections in adults are spontaneously cleared. Hence, the current disease burden of transfusion-transmitted hepatitis B infection is very small.

Following the development of assays for HBsAg detection, it became apparent that HBV was not the primary causative agent of transfusion-associated hepatitis (TAH) and that some additional agent or agents accounted for at least 75% of cases.<sup>4</sup> It was initially thought that some or all of these non-B cases might be caused by the hepatitis A virus (HAV), the only other human hepatitis virus known at that time. However, HAV was definitively

identified in 1973<sup>5</sup> and when prospectively followed non-B hepatitis cases were retrospectively tested for antibody seroconversion to HAV, it was shown that none were HAV related. Thus, it became apparent that there was a third human hepatitis virus and this received the exclusionary designation, the non-A, non-B (NANB) agent, pending its further characterization. Although the NANB agent was shown to be transmissible to chimpanzees and was partially characterized in that model, it took more than a decade before the true nature of the NANB agent was revealed by the sophisticated approaches of the emerging field of molecular biology. In 1988, Houghton and coworkers at Chiron Corporation cloned the primary agent of NANB hepatitis, unraveled its molecular sequence, and expressed epitopes that now serve as the basis for highly specific serologic assays routinely used for blood donor screening.<sup>6</sup> The NANB agent was then renamed the hepatitis C virus (HCV) and was found responsible for at least 90% of cases of TAH.<sup>7</sup> The introduction of screening assays to detect antibody to HCV and subsequently the detection of amplified HCV RNA has resulted in a dramatic reduction in the incidence of transfusion-associated hepatitis C that is now calculated to be approximately one infection per 1.5 to 2.0 million transfusions.<sup>8</sup> In addition, virus inactivation procedures for pooled plasma products, including clotting factor concentrates, has rendered these formerly high-risk products virtually hepatitis-free. Efforts are now under way to develop virus inactivation procedures for cellular blood components. Although this will be more difficult, such an accomplishment would presage the end of TAH, as well as many other transfusion-transmitted infections.

**Incidence of Transfusion-Associated Hepatitis**

The incidence of TAH in the United States was formerly based on data from prospective studies, but as the incidence decreased to rates less than 1%, it became logistically and economically unfeasible to conduct prospective studies with sufficiently large numbers of subjects to achieve a statistically valid incidence. The incidence of TAH is now derived from mathematical modeling

based on 1) the incidence of HBV and HCV infections among repeat blood donors and estimates of the window period between exposure to these agents and the first detectable marker of infection in recipients (incidence-window period model) or 2) the yield of new infections based on detection of nucleic acid in the absence of antibody [nucleic acid amplification test (NAT)-yield model]. The need to utilize mathematical models is a reflection of the success of blood bank programs in interdicting donors with high-risk behavior and in detecting those with serologic or molecular markers of hepatitis virus infection.

Before mathematical modeling, determination of the incidence of TAH required prospective studies to detect biochemical evidence of liver disease because 75% of cases of TAH are clinically inapparent and because fewer than 10% of overt cases are reported to the transfusion service. Before 1970, a prospective study of patients undergoing open-heart surgery at the National Institutes of Health (NIH)<sup>9</sup> showed that the incidence of TAH exceeded 30%. The introduction of an all-volunteer donor system and first-generation assays for HBsAg dramatically reduced the incidence of TAH to the range of 10% to 12%.<sup>3</sup> That incidence persisted through the 1970s despite increasingly sensitive tests for HBsAg, but declined from 10% to 6% from 1980 to 1987.<sup>9</sup> By 1989, the cumulative effect of more stringent donor questioning regarding high-risk behaviors, the introduction of human immunodeficiency virus (HIV) and anti-HBc assays as surrogate markers for NANB hepatitis, the routine virus inactivation of plasma products, and the more judicious use of blood in the wake of the AIDS epidemic served to decrease the incidence of TAH to approximately 4%. The introduction of specific anti-HCV assays in 1990 led to a further marked decline in the incidence of TAH to approximately 1%.<sup>9</sup> Testing of stored donor and recipient serum revealed that first-generation anti-HCV assays could have prevented 80% of cases of TAH that occurred before 1990 and that second-generation assays, introduced in 1992, could have prevented 90% of cases.<sup>7</sup> On the basis of the prevalence of HCV in the donor population just before anti-HCV testing, it is estimated that the introduction of serologic tests for anti-HCV prevented 40,500 cases of TAH per year (111 cases per day) in the United States alone.

Mathematical modeling conducted before the implementation of routine NAT testing of donor blood estimated the risk of HCV transmission to be 1 case per 100,000 transfusions and the risk of HBV transmission to range from 1 in 65,000 to 1 in 150,000 transfusions.<sup>8</sup> After the implementation of minipool (MP)-NAT testing, the calculated risk of HCV infection was reduced to 1 in 1.5 million transfusions.<sup>8</sup> Individual donor (ID)-NAT testing could decrease that risk further, but the benefit would be marginal given the already low frequency with minipool donor testing. The current risk of transfusion-transmitted HBV infection is still calculated to be relatively high (approximately 1:150,000 to 1:175,000), but that is based on anti-HBc seroconversions and an adjustment for missed HBsAg detection and does not represent clinical or even biochemical hepatitis.<sup>8</sup> Actual transfusion-associated hepatitis B cases appear to be quite

rare. Nucleic acid testing for HBV has not been implemented as of this writing, but individual NAT for HBV would decrease the current seronegative window by 7 to 25 days, depending on the sensitivity of the HBsAg assay, and might bring the risk of HBV infection into the same range as the risk of HCV infection.<sup>8</sup> Blood transmission of hepatitis A or hepatitis E in the developed world is so rare as to be reportable. It is still unclear whether there is a human hepatitis beyond the identified agents for hepatitis A to E. This is addressed later in this chapter.

## Hepatitis B Virus

### Historical Perspective

The first reported outbreak of percutaneously transmitted hepatitis was described more than a century ago, when 15% of German dockyard workers developed jaundice after smallpox vaccination with lymph derived from convalescent persons.<sup>10</sup> Although HCV cannot be excluded, the most likely responsible agent was HBV. However, HBV infection probably has afflicted humankind since ancient times, judging by the extraordinary prevalence of the virus in Asia and Africa, where it has been perpetuated by perinatal and percutaneous transmission.

In the third and fourth decades of the last century, with the widespread use of syringes, percutaneous spread of viral hepatitis regained notoriety. Reports followed describing jaundice among persons attending venereal disease<sup>11</sup> and arthritis<sup>12</sup> clinics. Because the disease seemed to be associated with the sharing of inadequately sterilized needles, it became known as “postinoculation jaundice.” Further support for a link between needle exposure and infection came from reports of severe hepatitis among children and British troops inoculated with convalescent measles and mumps sera<sup>13,14</sup> and among recipients of yellow fever vaccine.<sup>15,16</sup>

During World War II, viral hepatitis research increased dramatically. Not only was “epidemic” hepatitis once again found to be common, but also the widespread use of blood transfusion led to the emergence of TAH first reported in 1943.<sup>17,18</sup> Subsequent investigations, including experimental human transmission studies, initially pointed to the existence of two viruses, with different modes of transmission, incubation periods, severity, and outcome.<sup>19-21</sup> Hepatitis B virus was held responsible for all cases of hepatitis following transfusion (“serum hepatitis” or “post-transfusion hepatitis”). The current terminology, *hepatitis B*, was proposed in the late 1940s and adopted by the World Health Organization (WHO) in the mid-1970s.

Proof that HBsAg was associated with a transmissible infectious agent came from the earliest studies, employing an insensitive immunodiffusion test.<sup>22</sup> Approximately 75% of recipients of HBsAg-positive donor blood were found to have either hepatitis or serologic markers of HBV infection, although hepatitis also was noted in recipients of HBsAg-negative donor blood. Testing for HBsAg evolved from agar gel diffusion to counter-electrophoresis, to radioimmunoassay and finally, to enzyme

immunoassay (EIA). Testing of all blood donations for this serum marker was made mandatory by federal regulation in July 1972 and third-generation HBsAg testing was mandated in 1975.

Prospective studies of TAH conducted during the 1980s and 1990s in the Far and Middle East, Europe, Canada, and Australia<sup>23-27</sup> showed that despite hepatitis frequencies ranging from 3% to 19%, few cases could be attributed to HBV. In the majority of studies, no cases of hepatitis B were identified, and hepatitis B accounted for 7% or fewer of total cases in three studies.<sup>23,26,28</sup> Widespread implementation of more sensitive assays for HBsAg and adoption of all-volunteer donor programs in developed countries has virtually eliminated clinically significant transfusion-associated hepatitis B.

## Molecular Virology

Hepatitis B virus is a 42-nm DNA virus composed of an outer shell (7 nm in width) and an inner core (27 nm in diameter). It belongs to a novel genus, *Hepadnavirus*, within the *Hepadnaviridae* family; HBV is designated hepadnavirus type 1. The outer coat material (or envelope), is composed of three virus-encoded polypeptides, collectively designated the hepatitis B surface antigen, and host-derived lipid components. The HBV envelope proteins can be detected in serum, not only as a part of the intact virus, but also as separate 22-nm spherical or tubular subvirus particles, that circulate in amounts that outnumber complete HBV particles by a factor of  $>10^4$ , that do not contain DNA, and that are highly immunogenic, but noninfectious. They, in fact, constituted the source for the first generation of plasma-derived HBV vaccines. Clinically, HBsAg is detected in serum by serologic assays, visualized in the cytoplasm of hepatocytes by electron microscopy, and identified in liver tissue of infected persons by its ground-glass appearance and by antigen-specific immunohistochemical staining.

The inner icosahedral nucleocapsid core of HBV is composed of the nucleocapsid protein known as the hepatitis B core antigen (HBcAg), the viral genome, and the viral DNA polymerase. The genome of HBV is a relaxed circular, partially double-stranded DNA, with a complete negative DNA strand (3200 nucleotides) and an incomplete positive DNA strand. The genomic information of the virus, present on the long strand of the DNA, comprises four highly overlapping (67%) open reading frames (referred to as *S*, *C*, *P*, and *X* genes), encoding the envelope (pre-S/S), core (pre-core/core), polymerase, and X proteins, respectively (Fig 46-1).

The *S* gene has three in-frame start codons and a common stop codon, dividing the gene into the preS1, preS2, and *S* regions encoding the *large* (L), *middle* (M), and *small* (S) envelope proteins, respectively, depending on where translation is initiated. The product of the *S* region is the HBsAg (major protein).<sup>29</sup> The M and S envelope proteins are found in both virions and subvirus particles, whereas the L proteins are found mostly in complete virions.

The *C* gene has two in-frame start codons. The core protein (HBcAg) is translated from the second start codon and expressed on the surface of the nucleocapsid. Translation from the precore

start codon produces a precore polypeptide that is posttranslationally cleaved into a soluble nonparticulate protein known as the hepatitis B e antigen (HBeAg).

The *P* gene, which encompasses 80% of the viral genome, encodes a single multifunctional protein with four distinct domains: a protein primer, a spacer, a reverse transcriptase (RT)/DNA polymerase, and an RNase H domain.

The *X* gene encodes a multifunctional transactivator of transcription (HBxAg), of both viral and cellular genes.<sup>30,31</sup> It has pleiotropic effects on the transcriptome of the infected cell, including signaling pathways, protein degradation, cell cycle checkpoints, cell death, and carcinogenesis.<sup>30</sup>

The HBV genome contains four promoters (preS1, preS2/S, C and X) that control transcription by the cell RNA polymerase II of six functionally co-terminal viral mRNAs.<sup>32</sup> The C promoter has a basic core promoter (BCP) sequence that acts as a binding site for a variety of cellular transcription factors (ie, HNF3, HNF4, SP1).<sup>33,34</sup>

HBV infects hepatocytes by binding a domain in the large surface protein to an unknown receptor and enters the cytoplasm by endocytosis. Once delivered to the nucleus, the plus-strand DNA is completed by the DNA polymerase and the HBV genome is converted into a covalently closed circular DNA (cccDNA) that acts as the template for transcription of five viral RNAs. These comprise two 3.5 kb precore (translated into the precore polypeptide, which is further processed at both ends to the HBeAg) and pregenomic RNAs, two 2.4 and 2.1 kb mRNAs for the surface proteins and a 0.7 kb mRNA for the X protein.

The pregenomic RNA, which functions as the messenger RNA for translation of the HBcAg and the polymerase, is also the template for reverse transcription. A stem-loop near the 5' end of the pregenomic RNA contains a packaging signal ( $\epsilon$ ) and is used by the RT domain of the viral polymerase to prime minus-strand DNA synthesis with concomitant packaging by the nucleocapsid protein into immature cores. Inside the immature cores, a new minus-strand HBV DNA is produced through reverse transcription of the pregenome followed by the synthesis of a new plus-strand DNA. Maturation of the nucleocapsid before plus-strand synthesis is complete results in the partially double-stranded genome.

The cccDNA persists as a stable chromatinized episome in the cell nuclei, appears to have a very long half-life, and is very resistant to antiviral therapy. Its stability along with the long half-life of hepatocytes implies that once HBV infection has occurred it may continue for life, even after apparent resolution.<sup>35</sup>

## Genetic Variation of HBV

HBV can be classified into eight genotypes (A to H) based upon an inter-group divergence of  $\geq 8\%$  in the complete nucleotide sequence.<sup>36</sup> There is growing evidence suggesting that HBV genotypes influence clinical outcomes, HBeAg seroconversion rates, mutational patterns in the precore and core promoter regions, and response to antiviral therapy.<sup>37,38</sup> The prevalence of specific genotypes varies geographically, with genotype A found mainly

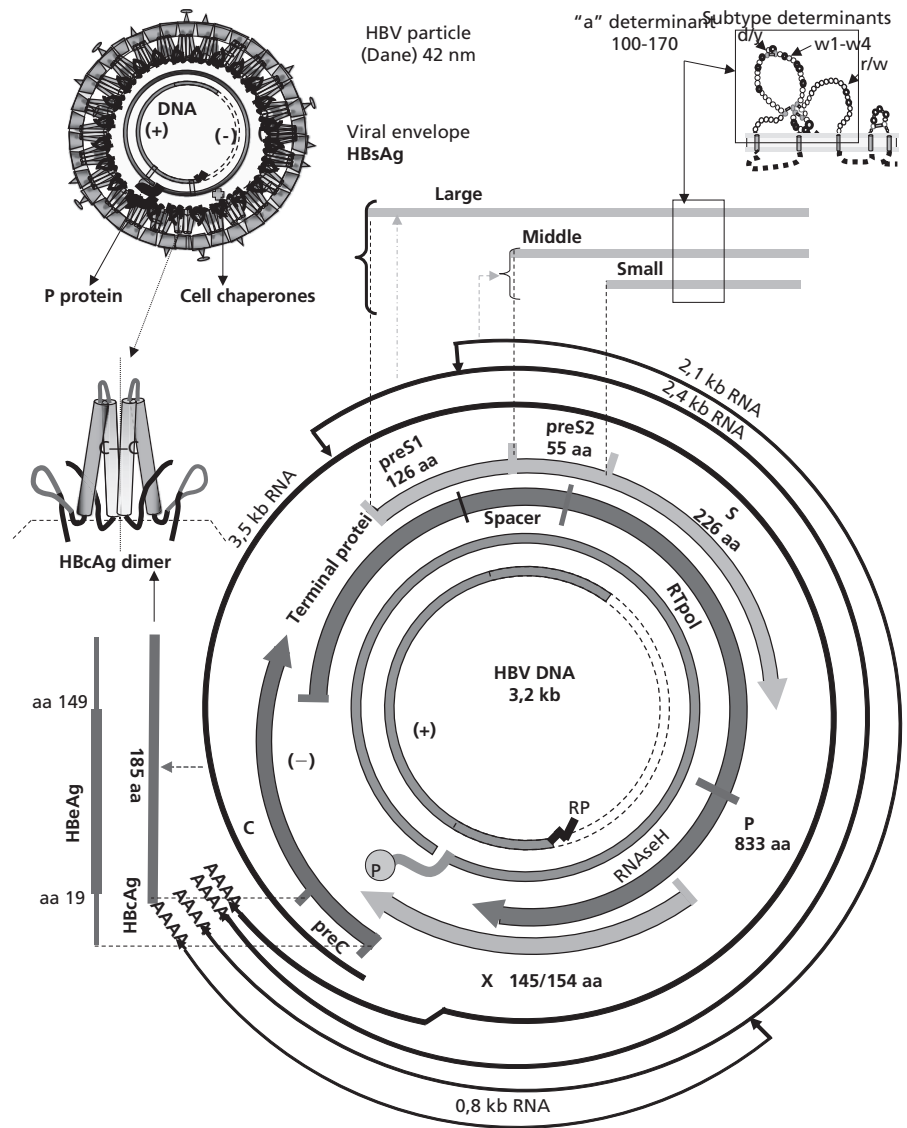


**Figure 46-1.** Genomic structure and translated proteins of HBV. The inner circles represent the full-length minus-strand DNA, with the terminal protein attached to its 5' end, and the incomplete plus-strand DNA of the HBV genome. The shaded lines represent the four overlapping open reading frames (ORF) S, C, P, and X and their corresponding encoded proteins and detailed structure of the main HBV antigens encoded (HBcAg, HBeAg, and HBsAg). The outermost thin black lines represent the 3.5, 2.4, 2.1, and 0.7 kb mRNA transcripts, which are all terminated near the poly-A signal. Details of the HBcAg dimers that assemble into the inner core and the structure of the HBsAg domain, common to all HBV surface proteins, containing the main "a" are also shown (see also text).

in Northern Europe, North America, India, and Africa; genotypes B and C in Asia; and genotype D in Southern Europe, the Middle East, and India. All known genotypes have been found in the United States, with prevalence of genotypes A, B, C, and D of 35%, 22%, 31%, and 10%, respectively, while genotypes E to H represent 2%.<sup>39</sup>

The HBV viral polymerase lacks proofreading activity, resulting in an estimated error rate of  $10^{-4}$  to  $10^{-5}$  nucleotide substitutions per site/year. Combined with a high turnover rate (up to  $10^{11}$  virions/day), this leads to the accumulation of a swarm of closely related genomic sequences, referred to as quasispecies. Although mutations occur randomly along the genome, the overlapping open reading frames limit the number and location of viable mutations.<sup>40-42</sup> Mutants may become selected according to their relative fitness when environmental conditions change

[ie, during antiviral treatment or hepatitis B immune globulin (HBIG) prophylaxis]. Most common and clinically relevant HBV mutants have been well characterized and affect the precore/core promoter, preS/S, and polymerase genes.<sup>40</sup>

#### Precore and Core Promoter Variants

HBeAg, which is not required for virus replication, seems to act as a decoy for the immune system, inducing tolerance. Viral clearance is usually associated with decreased HBeAg production followed by seroconversion to anti-HBe. However, documented severe liver disease in anti-HBe-positive patients with chronic hepatitis B (CHB), (HBeAg-negative CHB), led to the recognition of mutations in the precore region that prevent HBeAg synthesis. By far the most common of these mutations is a transition (G1896A), that creates a stop codon blocking translation of the

precore protein and HBeAg production. Less frequently, point mutations in the start codon can also abrogate HBeAg production. In the United States, precore variants have been found in 27% of patients with chronic HBV infection.<sup>39</sup> The most common core promoter variant involves a dual mutation A1762T, G1764A (TA change). In the United States, these variants have been found in 44% of patients with chronic HBV infection, and may also be associated with HBeAg-negative CHB.<sup>39</sup> Most patients with HBeAg-negative CHB harbor HBV variants in the precore or core promoter regions, have lower serum HBV DNA levels, and are more likely to run a fluctuating course.<sup>43,44</sup>

HBeAg-negative CHB has been estimated to account for 7% to 30% of patients with CHB worldwide, being predominant in Mediterranean countries and Asia.<sup>45,46</sup> The most common HBV variant associated with HBeAg-negative CHB, G1896A, usually occurs in HBV genotype D infection (more prevalent in the Mediterranean basin) and is rare in genotype A infections (more common in the United States and northwestern Europe).

Although increasing data support an association between core promoter variants and more severe liver disease, hepatic decompensation and hepatocellular carcinoma (HCC), large-scale longitudinal studies are needed to clarify this association. Precore and core promoter variants have also been described in association with fulminant acute hepatitis B, and have been associated with higher resistance to interferon and nucleoside analog therapy.

### Pre-s/s Variants

The major B-cell neutralizing epitope of HBsAg resides in the “a” determinant region located at amino acid positions 124 to 149. Antibodies to the “a” determinant confer protection against all HBV serotypes. HBV S gene mutants have been described in infants infected with HBV despite an adequate anti-HBs response to HBV vaccination (vaccine escape mutants). The most common mutation involves an amino acid substitution at codon 145 (G145R) in the “a” determinant of HBsAg. Recurrent hepatitis B after liver transplantation despite HBIG prophylaxis has also been associated with this mutation. Finally, in rare instances, mutations in the HBV genome downregulate S gene expression or produce aberrant surface proteins undetectable by conventional HBsAg assays, despite ongoing HBV replication. Such “occult” HBV infections may have an impact on blood transfusion, organ transplantation, and diagnosis of cryptogenic liver disease.

### Polymerase Gene Mutations

The RT/DNA polymerase region has five conserved domains: A, B, C, D, E, with the putative catalytic domain believed to reside in the YMDD locus in domain C. Naturally occurring polymerase gene mutations are rare, but many mutants are selected in patients receiving treatment with oral nucleos(t)ide analogs.

### Profile of Serologic and Molecular Markers During Hepatitis B Virus Infection

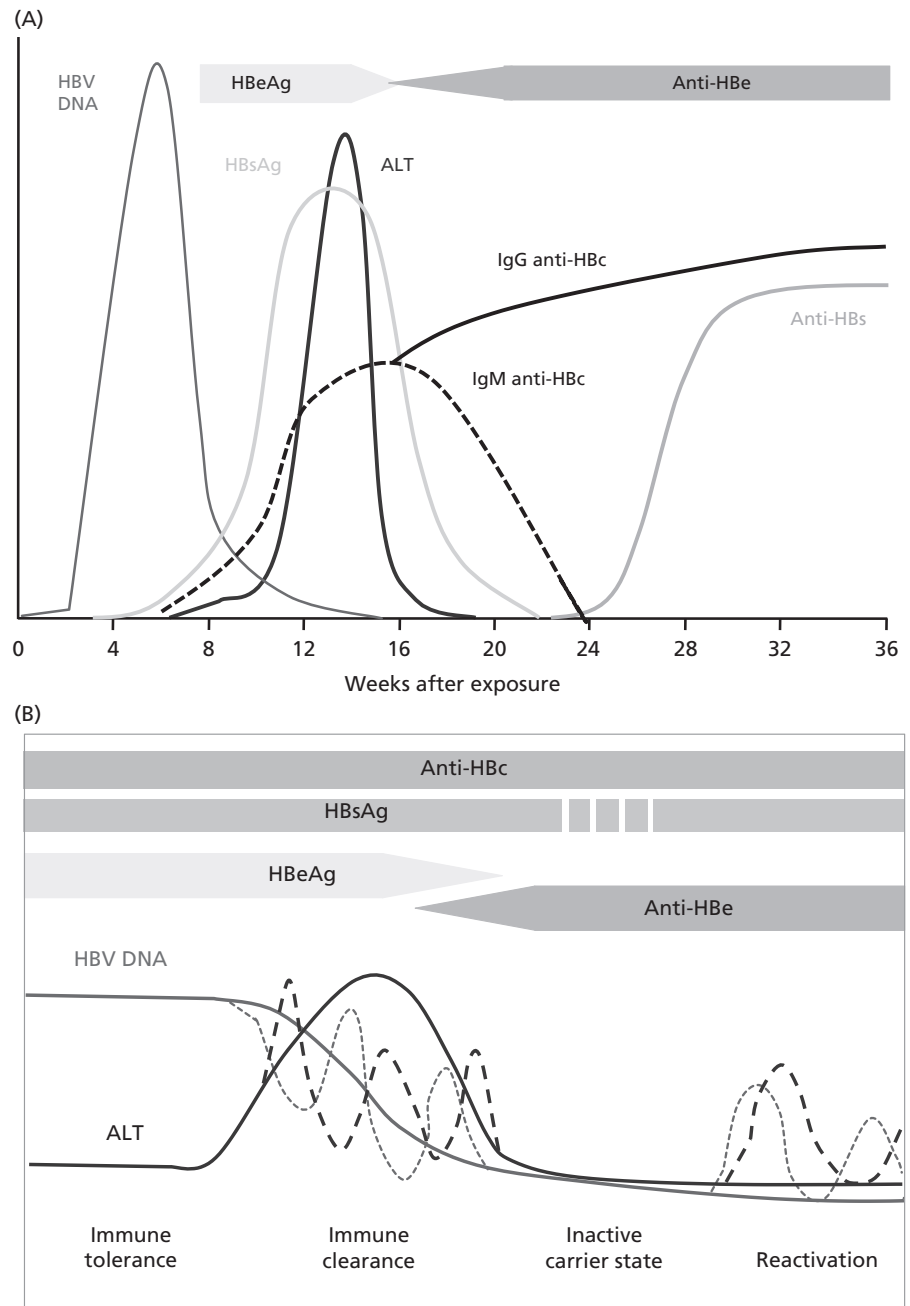
Infection with HBV is associated with characteristic changes in the serum levels of hepatitis B antigens and antibodies and HBV

DNA, which define different clinical states.<sup>47</sup> Acute infection is characterized by the appearance of HBsAg, HBeAg, and HBV DNA 1 to 10 weeks after exposure, followed soon thereafter by anti-HBc (initially of the IgM and then the IgG class). After exposure, there is a phase, lasting approximately 3 weeks (1 to 6 weeks), in which serum HBV DNA is intermittently detectable at low levels, followed by a “ramp-up” phase during which HBV DNA increases rapidly with an estimated doubling time of 2.56 days.<sup>48</sup> The period between exposure and first appearance of detectable HBsAg is referred to as the *window period* (WP). During this WP, the duration of which depends on the sensitivity of the serologic assay, HBV DNA is the only marker of HBV infection. Hepatitis is defined by the observed onset of increased alanine aminotransferase (ALT) activity, 25% or fewer of the cases being associated with jaundice. Usually, HBeAg and HBV DNA, detectable by hybridization assays with limits of  $10^5$ - $10^6$  copies/mL, disappear early, and HBsAg titers decline as the ALT peaks. Soon thereafter, the enzyme values begin to decline. What remains is IgM anti-HBc, which, after 6 months or longer, is replaced by IgG anti-HBc, the latter persisting for life in decreasing titers. During the interval between loss of HBsAg and development of anti-HBe and anti-HBs, which may extend for weeks or months, IgM anti-HBc may be the sole marker of acute HBV infection. The usual recovery sequence begins with the loss of replicative markers (HBeAg and HBV DNA), seroconversion to anti-HBe and disappearance of HBsAg, return of ALT levels to normal, and, finally, appearance of anti-HBs, which conveys immunity to reinfection [Fig 46-2(A)]. Although recovery is associated with disappearance of serum HBV DNA as determined by insensitive assays; when very sensitive assays [polymerase chain reaction (PCR) or transcription-mediated amplification (TMA)] are used, HBV DNA may remain detectable (sometimes intermittently) for many years.<sup>49</sup>

Persistence of HBsAg and usually of HBeAg beyond 6 months defines chronic infection (which occurs with variable frequency depending on the age at infection and immune status of the host). HBV DNA is usually detectable with insensitive assays (levels  $\geq 10^5$ - $10^6$  copies/mL) as well as anti-HBc. HBeAg may persist for years or decades during chronic infection and its presence is usually associated with high levels of serum HBV DNA and active liver disease. Over time during chronic infection, spontaneous or treatment-induced seroconversion to anti-HBe occurs, usually associated with a marked decrease in serum HBV DNA and remission of liver disease. Some patients with mutations that prevent HBeAg production, however, continue to have active liver disease despite anti-HBe seroconversion (HBeAg-negative CHB) [Fig 46-2(B)].

There exists a subset of patients with “occult HBV infection” defined as being HBsAg negative with persistent HBV DNA detectable by PCR, in the presence (80%) or absence (20%) of anti-HBc with or without anti-HBs. Most of these patients have very low or only intermittently detectable serum HBV DNA levels; although HBV DNA is usually present in the liver. Infection with preS/S HBV variants accounts for a small number of these cases. HBV transmission from recently infected blood donors

**Figure 46-2.** Clinical, serologic, and virologic course of acute and chronic HBV infection. (A) Schematic profile of serologic and virologic markers of a typical acute resolving hepatitis with the average timing of detection of serum HBV DNA, HBsAg, and HBeAg and their corresponding antibodies, with respect to the ALT elevation reflecting immune-mediated liver cell destruction after HBV replication (as reflected by serum HBV DNA levels) has been mostly controlled by noncytolytic mechanisms. (B) Course of chronic hepatitis B after perinatal or early-childhood-acquired infection. The four distinct phases shown are: immune tolerance (high-level HBV replication with normal ALT levels), immune clearance (high ALT level, lower HBV DNA levels and frequent hepatitis flares, depicted with dashed lines for both HBV DNA and ALT, which may lead to loss of HBsAg and anti-HBe seroconversion), inactive carrier state (normal ALT, presence of anti-HBe and low or even undetectable HBV DNA), and reactivation phase after many years of inactive carrier state (lower and fluctuating HBV DNA and ALT levels in the presence of anti-HBe), commonly associated with selection of core or precore HBV variant (see text for further details).



with precore mutants, who never developed detectable HBsAg, has been described in Japan.<sup>50</sup> Most cases of occult HBV infection represent late stages of persistent HBV infection associated with a strong immune response from the host, and/or viral interference because of coinfection with HCV.<sup>51</sup> Among blood donors and the general population in developed countries cryptic HBV infection is rare,<sup>52</sup> but it is frequently detected in countries that have a high prevalence of HBV (~10%-15%).<sup>53</sup>

### Epidemiology and Prevention

According to WHO estimates, 2 billion people have been infected with HBV, of whom 350 million are chronic carriers,

and 4 million new infections occur annually. HBV is readily transmitted by percutaneous or transmucosal transfer of blood or body fluids (eg, semen, cervicovaginal fluid, saliva). Three patterns of infection exist. In developing countries of Asia and sub-Saharan Africa, the disease is transmitted predominantly during the perinatal period by carrier mothers or, less commonly, through horizontal household transmission during infancy or early childhood. In developed countries, infection occurs mainly in adolescents and young adults through intimate (usually sexual) contact with chronic carriers or by percutaneous exposure (injection drug use, tattoos, multiple transfusions, accidental needlestick exposure, etc). There is a striking inverse

relationship between age at infection and the risk of chronicity<sup>54,55</sup>; the chronicity rate is ~90% for perinatal infection, 20% to 50% for infection acquired before 5 years of age and 1% to 5% for adult acquired infections. Hence, the prevalence of HBV carriers varies from 0.1% to 2% in low-prevalence areas (United States, Canada, Western Europe, Australia), to 2% to 5% in intermediate-prevalence regions (Mediterranean countries, Japan, Central Asia, Eastern Europe, Middle East, and Latin America), to 7% to 20% in high-prevalence areas (Southeast Asia, China, sub-Saharan Africa).

Several studies undertaken soon after the implementation of HBsAg screening showed that it was more common in males, in African and Asian Americans, and in paid donors. In addition, the HBsAg prevalence was higher among certain population groups (injection drug users and promiscuous men who have sex with other men).<sup>56</sup> A study of the risk of blood-borne viral infections at five blood donor centers from 1991 to 1996, involving 1.9 million donors showed no reduction in the frequency of HBV infection (0.2% HBsAg-positive first-time donors during this 6-year period).<sup>57</sup> A more recent study has shown a progressive decline from 1995 to 2002 to a prevalence of 0.07% among first-time donors.<sup>58</sup> However, changing blood donor demographics as a result of immigration of people from areas of moderate to high endemicity may increase HBV prevalence again.

Among the preventive measures for HBV infection, adequate information to HBV carriers about transmission mechanisms and counseling to avoid spread should be provided (use of condoms during sexual intercourse with occasional partners; not sharing toothbrushes and razors; covering open cuts and scratches; cleaning blood spills with bleach; not donating blood, sperm, or organs; and vaccinating regular sexual partners and household members). A highly effective HBV vaccine (recombinant HBsAg), confers protection to all HBV genotypes and subtypes in >95% of immunocompetent individuals. In high- or intermediate-prevalence areas vaccination campaigns of all newborns (along with HBIG in children born to HBV carrier mothers) and children under 12 years of age, have been widely implemented during the last 10 to 20 years so that, in many countries most individuals <25 years old are protected from HBV infection. Recommendations for vaccination have been established in recent Centers for Disease Control and Prevention (CDC) and Advisory Committee on Immunization Practices (ACIP) guidelines.<sup>59,60</sup>

## Screening Tests and Detection Systems

### Serologic Assays

After the donor selection process, testing for HBsAg remains the first-line screen for HBV. Since its introduction in 1970, HBsAg assays have increased their sensitivity by more than 2 log<sub>10</sub>. During the 1970s, extensive prospective studies (undertaken to evaluate the frequency of hepatitis and the efficacy of immune globulin prophylaxis) that coincided with the appearance of serologic tests for HBsAg, documented the impact of screening tests with greater assay sensitivity. For example, in a study

performed at the blood bank of the NIH, the frequency of transfusion-associated hepatitis B was 4.8% with the gel diffusion test, 3.7% with counter-electrophoresis (CEP) screening, and 0.6% after screening by radioimmunoassay (RIA) became available.<sup>3</sup> Currently licensed tests are third-generation EIA or chemiluminescent immunoassays (ChLIA), with analytical sensitivities varying from 0.13-0.62 to 0.07-0.12 ng/mL, respectively, as assessed in seroconversion panels.<sup>48,61</sup> The infectious WP for licensed EIA tests has been estimated at 37 to 50 days, and 33 to 44 days for the recently licensed ChLIA tests.<sup>62</sup> Aside from the WP, HBsAg immunoassays may fail to detect HBV DNA positive donors infected with some preS/S mutants, acutely infected donors with transient antigenemia and chronic carriers with occult HBV infection.<sup>50, 51</sup>

Testing for anti-HBc, originally introduced in 1986 as a surrogate marker for NANB infection, was retained because of its potential value for the detection of HBV-infected donors. The effectiveness of screening for anti-HBc as a means to reduce HBV transmission may be very low, because of its low specificity, and the deferral of a large proportion of otherwise eligible donors; the vast majority of repeatedly reactive anti-HBc-positive units also carry high-titer anti-HBs and are consistently negative for HBV DNA. Although cases of hepatitis B infection have been described in recipients of HBsAg-negative, anti-HBc-positive-only blood, their prevalence is quite low (and often represent false-positive test results) thus limiting the value of anti-HBc screening in low-prevalence populations.<sup>63</sup> Furthermore, in contrast to studies from high-prevalence regions, where HBV DNA has been identified by PCR in 5% to 25% of anti-HBc-only donors,<sup>64,65</sup> in low-prevalence areas (United States, Canada, Western Europe), HBV DNA has been detected in only 0.5% to 3.5% of anti-HBc-only blood donors<sup>66-68</sup> and infectivity of these low-level HBV DNA-containing units has not been well established. Nonetheless, anti-HBc screening has been implemented in several low-prevalence countries (United States, Germany, United Kingdom, France, Canada, New Zealand), often along with HBV DNA testing, because HBV infection continues to pose the greatest residual risk of transmission of all major blood-borne viruses.

### Molecular Assays for HBV DNA

Qualitative and quantitative tests for HBV DNA in serum (and liver tissue) have been developed to assess HBV replication. The sensitivity limit and dynamic range of these assays depends upon the techniques used. These vary from several hybridization assays (detection limit 10<sup>3</sup>-10<sup>6</sup> copies/mL) to the most sensitive amplification procedures such as real-time PCR and TMA assays. Commercial assays based on the latter procedures are available and have excellent performance with low-end detection limits of 5 to 50 IU/mL (25-250 copies/mL).<sup>48,69,70</sup> Although the main clinical utility of quantitative tests for HBV DNA is the selection and monitoring of patients for antiviral treatment, sensitive (quantitative or qualitative) tests have also been developed in semiautomated or automated platforms for HBV NAT of large numbers of blood donors, often in multiplexing formats for



simultaneous detection of HCV RNA, HIV RNA, and HBV DNA in MP-NAT (minipool) or ID-NAT (individual donor).<sup>71</sup> The use of NAT testing of blood donors for HBV DNA is intended to shorten the infectious WP and to detect occasional cases of occult HBV infection. Transmission from occult late-stage chronic carriers may contribute little to HBV transfusion risk in countries performing routine anti-HBc screening, but might be more relevant in areas with intermediate prevalence where anti-HBc screening is not performed. Nonetheless, the infectivity of such donors (3% in a look-back study conducted in Japan),<sup>50</sup> and the clinical significance for the donors remain largely unknown and are being actively investigated.<sup>72</sup> Most of the residual risk (RR) of transfusion-transmitted HBV comes from recently infected individuals who donate during the infectious WP and this risk, in the absence of NAT, has been estimated to range between 0.75 and 200 per million donations from repeat donors in areas where anti-HBc screening has not been implemented and between 0.91 and 8.5 per million donations in areas screening for anti-HBc (3.6 per 10<sup>6</sup> donations in the United States).<sup>73</sup> However, implementation of NAT for HBV remains controversial because of uncertainties in the length of the infectious WP on the incidence of infection among first and repeat donors and on the minimum inoculum size required for transmission. In addition, the new HBsAg tests may be 10-fold more sensitive than current EIA tests and may have already shortened the WP significantly. A mathematical model to estimate the potential impact of HBV NAT testing in reducing WP and relative risk according to the analytical sensitivity of NAT assays, pool size, doubling time of the virus, the incidence rate and back-extrapolation to a putative infectious dose of one DNA copy per 20 mL (the plasma volume of an additive solution red cell concentrate) has been proposed.<sup>62</sup> According to this model, ID-NAT for HBV would reduce the WP to 20 days (a 50% reduction from current estimates).

### Pathogenesis and Mechanisms of Viral Persistence

HBV is not directly cytopathic and both liver injury and control of HBV replication appear to involve HBV-antigen-specific cytolytic T cells (CTL) and CD4+ T cells, as well as cytokines released by both virus-specific T cells and nonspecific lymphocytes.<sup>74,75</sup> The early immune response to HBV does not appear to involve a significant direct innate immune response during the first weeks of infection.<sup>76</sup> Nonetheless, HBV DNA can be cleared from serum and liver before detectable T-cell infiltration and liver injury. Downregulation of HBV replication, with removal of both single-stranded intermediates and cccDNA from the cytoplasm and nucleus, is mediated primarily by interferon- $\gamma$  (IFN- $\gamma$ ), presumably released by HBV-specific CD8+ T cells as well as other activated cells.<sup>77,78</sup> Clearance of most HBV DNA from blood and liver is followed by development of multispecific CD4+ and CD8+ T cells, cytolysis of HBV-infected hepatocytes, and ALT elevation. A broad and vigorous multispecific CD4+ and CD8+ T-cell response is essential for recovery as evidenced by in-vivo depletion studies in the chimpanzee model.<sup>79</sup>

Viral persistence occurs mainly in vertically transmitted HBV infection (>90% of newborns to HBeAg-positive carrier mothers with high HBV DNA levels) presumably because the immune system of neonates is not fully developed, allowing development of immune tolerance to nucleocapsid proteins induced by HBeAg. The mechanisms of chronicity in settings other than perinatal transmission may involve qualitative and quantitative differences in both innate and adaptive immune response.<sup>80</sup> The T-cell immune response not only plays a role in viral control and recovery, but also contributes to liver damage in acute and chronic hepatitis. Flares of disease activity during the immune clearance phase of CHB coincide with increased serum interleukin (IL)-12 levels, increased HBV-specific CD4 and CD8 T-cell responses, and recruitment of large numbers of neutrophils, natural killer (NK) cells and activated bystander lymphocytes. These nonspecific immune cells are believed to mediate most of the liver damage.

### Clinical Spectrum and Natural History of HBV Infection

#### Acute Hepatitis B

The spectrum of clinical manifestations of acute HBV infection varies, depending on host factors (age, immune status, and underlying liver disease), ranging from subclinical or anicteric hepatitis to icteric hepatitis and, in some cases, fulminant hepatic failure.

After an incubation period of 1 to 3 months, approximately 70% of patients with acute HBV infection have subclinical hepatitis. In those with symptomatic disease, the clinical picture is indistinguishable from that of other viral hepatitis with symptoms and jaundice lasting 1 to 3 months. The disease may be more severe in patients coinfecting with other hepatitis viruses or with underlying liver disease. Fulminant hepatitis is unusual (0.1 to 0.5%). Laboratory testing during the acute phase reveals elevation of aminotransferases (values up to 1000-2000 IU/L), and bilirubin, which usually return to normal within 1 to 4 months in patients who recover. Despite clinical, analytic, and serologic recovery from acute infection, traces of HBV DNA remain detectable in blood and/or liver tissue by PCR for many years, suggesting that eradication of HBV rarely occurs and that latent infection is kept under control by the immune system.

Data from several prospective studies conducted during the early 1970s indicate that transfusion-transmitted hepatitis B begins after an average incubation period of 11 to 12 weeks.<sup>81</sup> In general, the clinical symptoms of the different types of viral hepatitis are indistinguishable from one another, although symptoms are more frequently absent among persons with HCV than among those with HBV infections. The biochemical dysfunction also tends to be more severe with HBV disease than with transfusion-transmitted HCV, with higher mean ALT values (705 vs 471 U/liter) and a higher frequency of jaundice (60% vs 25%).<sup>81</sup> It is difficult to assess the disease course after transfusion because of the scarcity of well-defined cases studied prospectively. Although non-transfusion-related acute hepatitis B in immunocompetent adults leads to chronicity in 1% to 5%

of cases, too few large-scale, prospective studies have been conducted to establish whether the frequency of chronicity in transfusion-associated cases is similar. The frequency of chronic HBV infection is clearly influenced by the age at onset (neonates and infants vs adults or elderly people) and the immune status of the patient; it is debated as to whether the size of the inoculum (transfusion vs needlestick or sexual) also affects the likelihood of evolution to chronicity.

### Chronic Hepatitis B

Persistence of HBsAg beyond 6 months after acute infection is the hallmark of CHB. The overall view of the natural course of chronic HBV infection has changed with the recognition that HBV replicates throughout the course of CHB, that the immune response plays a major role in liver damage, and that the balance between immune response and viral replication is a dynamic process.<sup>82,83</sup>

The natural course of CHB consists of four phases [Fig 46-2(B)]. The first phase of immune tolerance is classically seen in patients infected at birth by vertical transmission and is characterized by the presence of HBeAg and high-level HBV DNA in serum but with normal ALT levels and minimal or absent liver inflammation. It may last 1 to 4 decades, a period during which spontaneous HBeAg seroconversion is infrequent. In childhood or adult-acquired HBV infection this phase is short-lived or absent. The second phase (immune clearance/HBeAg-positive CHB) is characterized by HBeAg persistence, high or fluctuating HBV DNA levels, persistent or intermittent ALT elevation, and active hepatic necro-inflammation. A hallmark of this phase is the occurrence of hepatitis flares, often accompanied by transient increases in serum HBV DNA, that are sometimes severe enough to lead to hepatic decompensation. The duration of the “immune clearance” phase, and the frequency and severity of the flares, correlate with the risk of cirrhosis and HCC.<sup>84</sup> An important outcome of this phase is spontaneous HBeAg clearance accompanied by anti-HBe seroconversion; clearance of HBeAg correlates with, but is not limited to, older age, higher ALT levels, and infection with HBV genotype B. The third phase (inactive HBsAg carrier state) is characterized by the presence of anti-HBe, normal ALT levels, low serum HBV DNA ( $<10^5$  IU/mL), and mild hepatitis with variable fibrosis, depending on the duration and severity of the prior immune clearance phase. The inactive carrier state may persist indefinitely, in which case the prognosis is generally favorable. This is supported by a long-term follow-up study of 296 HBsAg-positive, healthy blood donors in Italy,<sup>85</sup> whose survival was similar to that of 157 uninfected controls over a 30-year period, and in whom no episodes of hepatic decompensation occurred. However, 4% to 20% of inactive carriers have episodes of reversion to HBeAg positivity and 10% to 30% of inactive carriers have spontaneous reactivation of HBV replication and liver disease activity after years of quiescence. The fourth phase (HBV reactivation of inactive carrier state/HBeAg-negative CHB) is characterized by positive anti-HBe, detectable HBV DNA, elevated ALT, and active liver

disease.<sup>86</sup> Most cases represent reactivation after a period of the inactive carrier state of variable duration, but some progress directly from HBeAg-positive to HBeAg-negative CHB. Because this is a later phase in the course of CHB, patients are usually older and have more advanced liver disease. Most patients with HBeAg-negative CHB harbor precore or core promoter HBV variants and tend to have lower serum HBV DNA levels than during the immune clearance phase. A clinical course characterized by fluctuating ALT and HBV DNA levels. HBeAg-negative CHB, originally reported in Mediterranean countries,<sup>86,87</sup> is now being recognized with increasing frequency in all parts of the world.<sup>88,89</sup> Prevalence varies according to the predominant HBV genotype in different geographic regions.

Spontaneous clearance of HBsAg occurs in 0.5% to 1% of patients with CHB per year, usually in association with undetectable serum HBV DNA, normalization of ALT, and absence of liver disease activity. In the absence of cirrhosis or HCV coinfection, loss of HBsAg conveys a good prognosis. CHB is usually asymptomatic until the evolution to cirrhosis or HCC and the ensuing complications. Collectively, among patients with CHB, 40% develop cirrhosis, liver failure, and/or HCC. Cirrhosis develops during CHB with an annual incidence of 2% to 6% in HBeAg-positive and 8% to 10% in HBeAg-negative patients. Certain cofactors increase the risk, such as alcohol, HCV or HIV coinfection, high HBV DNA levels, and HBV genotype C.<sup>45,89,90</sup> Among patients with cirrhosis, persistent, high-level HBV DNA is associated with increased risk of hepatic decompensation and mortality. The annual incidence of HCC has been estimated to be  $<1\%$  for noncirrhotic carriers and 2% to 3% for patients with cirrhosis. Factors associated with increased risk of HCC include male gender, older age, a family history of HCC, core promoter mutation, HBV genotype C, HCV coinfection, and the presence of cirrhosis. Although preexisting cirrhosis is a strong risk factor, 30% to 50% of HBV-associated HCC occurs in the absence of cirrhosis.<sup>91</sup>

### Treatment

The aims of treatment of CHB are induction of sustained suppression of HBV replication and remission of liver disease activity, with the goal to prevent cirrhosis, hepatic failure, and HCC. Currently, six therapeutic agents have been approved for the treatment of adults with CHB in the US (IFN- $\alpha$ , and nucleoside or nucleotide analogs: lamivudine; adefovir; entecavir; and telbivudine), while other analogs are still undergoing trials (emtricitabine, tenofovir, and clevudine). Responses to treatment are categorized as biochemical (normalization of ALT levels), virologic (loss of HBV DNA and anti-HBe seroconversion), and histologic (reduction in activity and halting of fibrosis progression). Standard and pegylated IFN- $\alpha$  (which are given for finite periods, by subcutaneous injection), are associated with substantial side effects and have limited efficacy in inducing virologic response (35% in HBeAg-positive and 70% in anti-HBe-positive CHB). Despite its low initial efficacy, the advantage of pegylated IFN- $\alpha$  is that the virologic response in HBeAg-positive patients

who achieve anti-HBe seroconversion is sustained after discontinuation of therapy in up to 90% of cases and hence long-term therapy is not required. However, a sustained virologic response occurs in only 20% of HBeAg-negative CHB patients treated with pegylated IFN- $\alpha$ . Drug resistance does not occur in patients treated with IFN. In contrast to IFN, nucleos(t)ide analogs are given orally, have minimal side effects, and are more potent inhibitors of viral replication. However, they usually require long-term or indefinite administration (because of the low rate of sustained remission after withdrawal) and 30% to 70% develop resistant mutants after 2 to 5 years of treatment (<1% after 2 years with entecavir monotherapy).

Although there is no doubt that antiviral therapy modifies the natural history of chronic HBV infection—reducing liver-related morbidity, mortality, and HCC incidence—the current complexity of treating CHB (criteria for selection of candidates, choice of antiviral agent, monitoring of response, diagnosis and management of resistant mutants) has necessitated the development of evidence-based treatment guidelines for the management of CHB.<sup>91-93</sup> These guidelines will continuously evolve as new medications are added and as new drug interactions and resistance patterns emerge.

### HBV Summary

Hepatitis B virus, a hepadnavirus, causes acute and chronic infection, the latter evolving to cirrhosis, end-stage liver disease and HCC in 40% of cases, unless properly treated. It is transmitted perinatally and by blood and secretions and was a major cause of posttransfusion hepatitis until the risk was reduced by elimination of paid donors and by screening for HBsAg. A highly effective vaccine is available and vaccination campaigns in high and intermediate prevalence areas have been widely implemented in the last two decades. The near elimination of the risk of HBV transmission by transfusion has been a major part of the success of contemporary transfusion medicine. The small residual risk of HBV infection by blood transfusion will be further reduced with more sensitive serologic and molecular assays with the ultimate goal of eradicating this transmission mechanism from the epidemiology of HBV infection.

## Hepatitis D

Hepatitis D virus (HDV), also known as hepatitis delta virus, is a 35- to 37-nm defective RNA virus that contains a 1700-nucleotide RNA genome similar to that of plant satellite viruses and viroids. Hepatitis D virus is a hybrid virus that relies on HBV for its surface coat (HBsAg), which surrounds a nucleocapsid core expressing HDV antigen. HDV requires concurrent infection with HBV to support its replication and clinical expression.<sup>94</sup> Hepatitis B virus and HDV infections can occur simultaneously, or a patient with chronic HBV infection can become superinfected with HDV. Ultimately, the duration of HDV infection is determined by the duration of HBV infection because HDV invariably relies on HBV for its growth and expression. A diagnosis of HDV infection can be made by demonstrating antibody

(IgM or IgG) to HDV (anti-HDV), identifying HDV antigen (HDAg) in liver nuclei by immunohistochemical staining, or by detecting HDV RNA in serum by PCR or in liver by cDNA hybridization. The detection of HDAg in serum is difficult and therefore not a practical diagnostic marker.

Hepatitis D tends to segregate into two different epidemiologic patterns. In endemic areas, such as Mediterranean countries, HDV infection appears to be transmitted from person to person. In contrast, in nonendemic areas, such as northern Europe and North America, transmission of HDV infection appears to be confined to specific percutaneously exposed populations, such as drug users and hemophiliacs. Occasionally, even in nonendemic areas, protracted outbreaks of hepatitis D occur either by HBV-HDV coinfection or HDV superinfection. In nonendemic areas, such outbreaks have a tendency to amplify HDV infection in the community and to blur the epidemiologic distinction between endemic and nonendemic areas. The frequency of HDV infection, even in endemic areas of the world, has been declining.

The clinical features of hepatitis D are similar to those of hepatitis B, and most of the more severe outcomes of hepatitis B are caused by hepatitis D coinfection. In particular, superinfection of chronic hepatitis B with HDV often leads to more serious and rapidly progressive liver disease and can transform asymptomatic or mild chronic hepatitis B into severe chronic hepatitis and cirrhosis. In addition, fulminant hepatitis can follow HBV-HDV coinfection or HDV superinfection of an HBV carrier.<sup>95</sup>

Because HDV infection cannot occur in a patient who is immune to HBV, prevention of HDV infection is accomplished readily by HBV vaccination in persons susceptible to hepatitis B. For those already infected by HBV, prevention of superinfection is limited to mechanical efforts to avoid intimate contact with HDV-infected persons or HDV-contaminated needles or blood components.

### Hepatitis D and Blood Transfusion

Because, under ordinary circumstances, HDV infection cannot occur in the absence of HBV infection, the chance that a blood donor screened and found to be negative for HBsAg and anti-HBc could harbor HDV is exceedingly small. Before the advent of current screening techniques, transmission of HDV undoubtedly occurred and contributed to the previously identified high frequency of HDV infection in multitransfused persons, including those with hemophilia and thalassemia.<sup>96</sup> In a study of American patients with hemophilia A, 75% had a serologic marker of previous HBV infection, and 13% of them had antibodies to HDV.<sup>97</sup>

Although HDV is a blood-borne viral agent, contemporary, highly sensitive screening methods used in blood banks should be effective in reducing the frequency of transfusion-associated HDV hepatitis to a negligible level, at least in HBsAg-negative blood recipients. There is a very small residual risk for HBsAg-positive blood recipients because rare donors have occult HBV infection in which HBsAg is nondetectable despite the presence of HBV DNA. Theoretically, such donors could carry low levels

of HDV, though some level of HBsAg production would have to exist in order to support replication of HDV. Even this remote possibility of a donor with coexistent occult HBV infection and HDV infection would be interdicted in regions that screen donors for anti-HBc antibody.

## Hepatitis C Virus

### Historical Perspective

Before hepatitis C virus (HCV) was cloned, considerable knowledge of this agent was garnered from experiments in chimpanzees. In 1978, it was found that serum from patients with acute or chronic NANB hepatitis and from asymptomatic carriers could transmit infection to chimpanzees.<sup>98,99</sup> Although chimpanzees did not develop clinical illness, they had transaminase elevations and liver biopsy evidence of hepatitis after an appropriate incubation period. Animals inoculated with serum from patients with NANB hepatitis did not develop HBV markers, emphasizing the clear distinction between HBV and the agent of NANB hepatitis. In addition to elevations in transaminase levels, electron microscopy revealed that infected chimpanzees generally had characteristic tubular ultrastructures in hepatocyte cytoplasm.<sup>100</sup> These tubular structures, consisting of an electron-dense center and a double-unit membrane, represent proliferation of the rough endoplasmic reticulum, a change now shown to be induced by interferon. The tubules are an epiphenomenon of NANB/HCV infection and are useful indicators of such infection in chimpanzees because of their relative specificity. They are not induced during the course of HAV or HBV infection in chimpanzees and are not seen in human NANB/HCV infection.

The findings of elevated transaminase levels, light microscopic changes of hepatocellular damage, and electron microscopic evidence of cytoplasmic tubules made it possible to define NANB hepatitis in chimpanzees, even in the absence of a specific serologic marker. Using the chimpanzee as an infectivity model, it was shown that the cytoplasmic tubule-inducing NANB agent was sensitive to chloroform and other lipid solvents and thus that it contained essential lipid, presumably in its surface membrane.<sup>101,102</sup> Filtration studies subsequently showed that the NANB agent was 30 to 60 nm in diameter.<sup>103</sup> Among known viruses, only the flaviviruses and the hepadnaviruses (HBV, woodchuck hepatitis virus, duck hepatitis virus) are lipid encapsulated and fall into this size range. It was predicted that the NANB agent was a flavivirus and the subsequent cloning and characterization of HCV<sup>6</sup> proved this prediction correct.

Flaviviruses are a large and diverse family, formerly known as arboviruses (arthropod-borne viruses) and togaviruses. They include the dengue and yellow fever viruses, the rubivirus (rubella), and the pestiviruses (bovine diarrheal virus, hog cholera virus, and other mucosal disease viruses of animals). HCV represents a new genus in the family *Flaviviridae* and is most closely related to pestiviruses. Although there is only limited homology with the pestiviruses, the genomic structure and

hydrophobicity patterns are similar. The pestiviruses, like HCV, are characterized by blood-borne transmission and by lifelong viremia. Unlike dengue and yellow fever viruses, they do not have a mosquito vector.

### Cloning and Molecular Characterization

In the mid-1980s, a long series of experiments conducted by Houghton et al at the Chiron Corporation, in collaboration with Bradley at the CDC, culminated in the cloning and serologic characterization of HCV.<sup>6</sup> Using large volumes of plasma derived from a chimpanzee with chronic NANB hepatitis, these investigators extracted whole nucleic acid and made complementary DNA (cDNA) from RNA and single-stranded DNA templates. The cDNA was inserted into a gt11-phage expression vector and used to infect *Escherichia coli*. Expressed proteins were blotted and overlaid with serum from patients with NANB hepatitis. A single clone was identified that expressed an antigen reactive with chronic- and convalescent-phase serum from persons with NANB hepatitis, but not with serum from control subjects without infection or from patients with type A or type B hepatitis.<sup>6</sup> Cloned nucleic acid was used to generate probes that were shown to hybridize with RNA derived from the starting plasma, thus closing the loop between the cloned agent and the infectious plasma. Once the cloned agent of NANB hepatitis was molecularly characterized, it was designated HCV. Amplification of the reactive clones and expression of the genome in large cultures of yeast produced sufficient antigen to formulate radioimmune and enzyme-linked assays for the detection of antibody to HCV.

Characterization of HCV shows that the viral genome consists of single-stranded, linear RNA of 9.6 kb that is positively stranded and hence can serve directly as message for the synthesis of viral proteins.<sup>6,104</sup> The viral proteins are encoded from a single, large open reading frame that produces a polyprotein that is posttranslationally cleaved.<sup>104</sup> The genome (Fig 46-3) is characterized by a highly conserved, untranslated region at the 5' terminus (5'UTR) that is now used as the primary target for molecular amplification assays to detect HCV RNA and for viral genotyping. The 5'UTR contains an internal ribosomal entry site that is essential for viral protein synthesis and that initiates translation at the start codon of the large open reading frame.<sup>104</sup> Downstream of the 5'UTR are the coding regions for envelope proteins designated E1 and E2, followed by the coding region for the viral core (nucleocapsid). In the terminal portion of the E1 region is a hypervariable segment (HVR1), which is the most highly mutable region of the genome, presumably because its position in the viral envelope places it under a high degree of immune pressure. It is presumed that this region is the target for neutralizing antibody responses. Downstream from the structural genomic regions are a series of nonstructural coding regions designated NS2, NS3, NS4, and NS5. The full function of these regions is not known, but they have been shown to code for critical enzymes, including an NS3 protease, an NS3 helicase, and an NS5 polymerase.

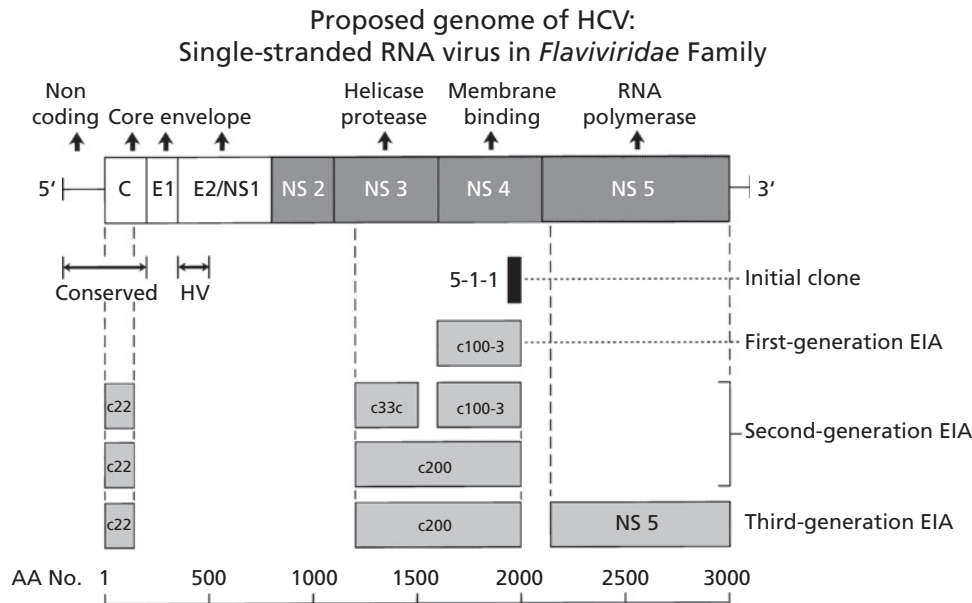


**Figure 46-3.** Proposed genome of hepatitis C virus (HCV). Functional equivalents and major antigens used in antibody detection assays. The structural organization of the HCV genome has been deduced from genomic sequencing and from parallels with other flaviviruses. The entire genome contains approximately 9500 bases coding for approximately 3000 amino acids. The 5' end has a highly conserved noncoding region, the one generally used for polymerase chain reaction amplification and presumed to have important regulatory functions. Downstream of the noncoding region are the regions coding for the structural elements, including the core or nucleocapsid (C) and the envelope (E1, E2/NS1). It is unclear whether NS1 is part of the envelope region or the first nonstructural gene. The 5' end of E2/NS1 contains a hypervariable (HV) region that mutates rapidly

There are six major genotypes of HCV that are designated in Arabic numerals 1 through 6 and each has been subtyped as designated by lowercase lettering. The genotypes differ from each other in nucleotide sequence by at least 15%. Genotypes 1a and 1b are the most prevalent forms in the United States, Europe, and Japan. In the United States, genotypes 1a and 1b are found in almost equal frequency, whereas in Japan genotype 1b predominates. Genotypes 2 and 3 are found as minor populations in the United States (10% genotype 2 and 6% genotype 3) and Europe. Genotype 3 is the predominant genotype in Southeast Asia. Genotype 4 appears isolated to Northern Africa (especially Egypt), and the Middle East, whereas genotype 5 has been localized to South Africa. Genotype 6 is rare and found predominantly in Japan. Although useful as epidemiologic tools and predictors of treatment response, the clinical significance of HCV genotypes is uncertain. Although many studies have claimed that patients with genotype 1 have more severe histologic outcomes and clinical disease, an equal number of studies have failed to show this association. The primary use of HCV genotypes is in the prediction of response to interferon-based therapies. In general, patients with genotypes 1 and 4 have an approximate 45% to 50% sustained virologic response to interferon plus ribavirin, whereas patients with genotype 2 or 3 infection have an

and probably plays a key role in the ability of the virus to escape neutralization. There follow a series of nonstructural genes (NS2-NS5) with enzymatic or membrane-binding functions. The initial clone discovered was from the NS4 region; the derived protein was designated 5-1-1. This was expanded to form the c100-3 antigen, the basis of the first-generation anti-HCV assay. Second-generation assays added the c22 core antigen and the c33c antigen from the NS3 region. These antigens increased the sensitivity of the second-generation assay approximately 20% over the first-generation test. The third-generation assay (pending licensure in the United States) adds an NS5 protein and reconfigures some of the earlier antigens. (Used with permission from Alter.<sup>105</sup>)

approximate 80% sustained virologic response rate that is akin to “cure” (see below).

## Detection Systems

### Surrogate Tests

Mounting evidence for the role of blood transfusion in the induction of chronic liver disease and continuing frustration in the development of a specific assay for the agent of NANB hepatitis prompted the adoption of surrogate (nonspecific) tests for the detection of NANB hepatitis virus carriers. These surrogate tests, ALT and anti-HBc, were implemented for routine donor screening early in 1987. Use of these tests evolved from retrospective analyses of prospective studies of TAH conducted by the Transfusion Transmitted Virus Study Group<sup>106,107</sup> and by the department of transfusion medicine at the NIH.<sup>108,109</sup> These studies found a threefold to fourfold increase in hepatitis risk among recipients of ALT-elevated or anti-HBc-positive blood compared to the rate among recipients of blood negative for these two indices. It was predicted from these studies that the adoption of ALT and/or anti-HBc tests for donor screening might result in a 30% to 40% reduction in TAH. The value of surrogate assays as an interim measure was validated when it was later shown that approximately one-third of donors with

confirmed anti-HCV were also positive for one or both of these surrogate markers.<sup>110</sup> A controlled prospective study conducted in Canada further validated the use of surrogate assays for the prevention of TAH.<sup>111</sup>

### Serologic Assays

Humoral responses to HCV are vigorous and are directed to a multiplicity of antigenic sites.<sup>104,112</sup> As shown in Fig 46-3, the original clone produced a protein designated 5-1-1, which was then expanded and fused to form the c100-3 antigen that served as the basis for first-generation anti-HCV assays. This early assay was highly effective in interdicting HCV carrier blood donors, but anti-c100-3 did not appear for a protracted period after exposure, creating a window of infectivity that ranged from 12 to more than 26 weeks. Some patients with HCV infection never developed antibody that was reactive in the first-generation assay. The second-generation assays added two critical epitopes to both the screening EIA and the confirmatory recombinant immunoblot assay (RIBA). These were a core protein, designated c22-3, and an NS3 protein, designated c33c. Antibodies to these new epitopes generally appear much earlier than anti-c100-3, and the window period in which a donor might be seronegative has considerably narrowed. In an NIH prospective study, 41% of persons with HCV infection had specific antibody detected with second-generation assays within 10 weeks of exposure, 80% had antibody within 15 weeks, and all patients had antibody within 6 months.<sup>113</sup> Third-generation EIA tests added an HCV antigen from the NS-5 region and have shown a marginal increase in sensitivity with a further reduction of the window period of approximately 1 week compared with second-generation assays. Because this increment in sensitivity would have minimal benefit in disease prevention, the use of third-generation anti-HCV assays has not been mandated by the US Food and Drug Administration (FDA).

Antibodies, particularly to 5-1-1, c100-3, and E1, may disappear spontaneously, during immunosuppression, or after successful antiviral therapy. Antibodies to c22-3 and c33c rarely disappear from persons with chronic infection and only infrequently disappear even from persons with apparent recovery. However, a minority of patients who clear HCV RNA also lose anti-HCV over time and are termed seroreverters; in most persons, some HCV antibodies persist for long periods, perhaps for a lifetime. The prevalence of HCV antibody, found with second-generation EIAs and confirmed with RIBA, among US blood donors ranges from 0.2% to 0.4%. Similar rates have been observed in Europe and higher rates are seen in Japan and other Eastern nations. These rates among highly selected blood donors are underestimates of the prevalence in the general population, which is estimated to be around 1%.

Because of nonspecific reactivity, it is important to confirm EIA reactivity with a supplemental assay. The only licensed supplemental test is a recombinant immunoblot strip assay that displays the key epitopes in a linear format on a nitrocellulose strip. With second-generation assays, approximately 40% of

EIA-reactive samples are confirmed by RIBA in a low-risk blood donor setting. Among high-risk populations or EIA-reactive donors who have elevated ALT levels, the confirmation rate is much higher (60% to 80%). In the initially licensed supplemental assay, approximately 30% of EIA-positive samples were classified as indeterminate because only one band appeared on the strip. A third-generation RIBA replaces the recombinant proteins c22-3 and c100-3 with synthetic peptides representing only a portion of the respective coding regions and replaces 5-1-1 antigen with recombinant NS5. This format resolves many RIBA-2-indeterminate patterns. Approximately 60% of RIBA-2 indeterminates are negative with RIBA-3, whereas 20% are RIBA-3 positive and 20% remain indeterminate. Thus RIBA-3 resolves approximately 80% of RIBA-2 indeterminate results. There is good, but not absolute, correlation between a positive RIBA result and NAT documentation of HCV RNA. Patients who have resolved HCV infection are RIBA positive, but repeatedly NAT negative. In contrast, almost all anti-HCV-positive persons who are HCV RNA positive also are RIBA positive. An alternative to RIBA testing is to go directly from a positive EIA screening assay to amplification of the viral genome by NAT or other technique. If HCV RNA is detected, there is no need to perform RIBA. However, if HCV RNA is negative, the RIBA assay should be performed to distinguish HCV-recovered subjects from those with false-positive EIA reactivity.

### Molecular Assays for Detection of HCV RNA

The most sensitive way to detect HCV is measurement of HCV RNA by means of NAT or another gene amplification technique. The NAT has shown that HCV RNA is almost universally detected in the early phase of HCV infection, generally within 1 to 3 weeks of exposure. Thus, HCV RNA detection precedes the increase in serum ALT level, sometimes by as much as 10 to 12 weeks. It also has been shown by means of quantitative NAT or branched DNA assay that the highest levels of HCV RNA appear early in the course of infection, generally preceding or coinciding with the first significant elevation in ALT level.<sup>105</sup> In persons who appear to recover from HCV infection, HCV RNA generally declines or disappears near the time of the peak in ALT elevation. However, in at least 75% of persons, HCV RNA persists, generally with associated fluctuating ALT elevations, but up to 30% of infected individuals have persistently normal serum ALT. These relationships imply that infectivity is probably greatest before signs or symptoms of acute hepatitis develop, that chronic HCV infection is more frequent than is indicated by ALT elevations alone, and that a proportion of persons with HCV infection may be true asymptomatic carriers with normal serum ALT levels and minimal histologic abnormalities in the liver.

The extreme sensitivity of NAT techniques and the long seronegative window between the first detection of virus and the first detection of anti-HCV has prompted the introduction of NAT into routine donor screening. NAT testing has been universally implemented in US blood establishments and in most blood centers in developed nations. Nucleic acid testing is being performed by two primary methods—standard

reverse-transcription PCR and TMA. Transcription-mediated amplification offers two advantages in the blood bank setting because extraction and amplification take place in the same tube under isothermal conditions and because the test lends itself to a duplex configuration, whereby HIV and HCV RNA can be measured simultaneously. A triplex TMA assay that also detects HBV DNA has been developed, but testing for HBV DNA is not mandated by the FDA at this time. As indicated earlier, the routine use of NAT has generated a better understanding of the dynamics of HCV and HBV viremia. With serial stored collections from commercial plasma donors who subsequently are found to be HCV-infected, it has been shown that HCV RNA usually is undetectable until the level increases precipitously to reach peak levels over a brief interval. This phase of rapid acceleration has been termed the ramp-up phase, and it usually occurs 2 to 4 weeks after exposure. During ramp-up, the doubling time of the virus has been calculated to be 13 to 17 hours.<sup>114</sup> The pattern of HBV viremia differs in that the ramp-up is more delayed and not as steep and in that the ramp-up is preceded by a persistent low-level of viremia. During the period of low-level HBV viremia before the ramp-up phase, possibly infectious donations can be missed at MP-NAT testing, but detected with ID-NAT testing. The doubling time for HBV during the ramp-up phase is calculated to be 2.8 days.

Another distinction between HBV and HCV testing is that MP-NAT dramatically decreases the infectious window for HCV (60 days, an 85% reduction) compared with detection of antibody to HCV. In contrast, MP-NAT (minipool) testing for HBV DNA reduces the window by only 6 days (13%) compared to detection of HBsAg; ID-NAT (individual donor) for HBV would decrease the window by to 25 days (55%) compared to current HBsAg detection,<sup>114</sup> but newer, more sensitive HBsAg assays will narrow the window between detection of HBV RNA and detection of HBsAg to as little as 1 week. In essence, to have a significant effect on HBV transmission compared to current practice, one would have to implement ID-NAT testing. For HCV, MP-NAT testing seems sufficient in most instances. A serologic test for HCV core antigen has been developed<sup>115</sup> and has shown sensitivity similar to that of HCV MP-NAT testing. This test may be an option for blood establishments that have not committed to NAT technology.

### HCV Cell Culture

The holy grail of HCV research has been to develop an in-vitro culture system. After 15 years of failure, this crusade now has come to fruition, spearheaded by the groundbreaking work of Wakita and collaborators in Japan<sup>116</sup> and subsequently expanded by several investigative groups.<sup>117,118</sup> The groundbreaking event was the isolation of a clone, designated JFH-1, derived from a single patient in Japan with fulminant hepatitis caused by HCV genotype 2a infection.<sup>116</sup> This full-length genome replicated without modification in a hepatoma cell line designated Huh-7. Replication was indicated by the finding of HCV RNA and HCV antigens within cultured liver cells, the secretion of intact viral particles, and the ability to passage the virus in culture and to

chimpanzees. Thus far, only this single JFH-1 isolate or its chimeras have been shown to grow in Huh-7 and other hepatoma cell lines. No genomic or structural difference has been identified to account for JFH-1's unique growth characteristics, nor is it known whether its high replicative capacity in vitro relates to the development of fulminant hepatitis in the infected patient. In regard to the latter, clones from other patients with fulminant hepatitis have not grown in tissue culture, nor did JFH-1 cause fulminant hepatitis in the chimpanzee. Building on the discovery by Wakita et al, Rice and coworkers<sup>117</sup> constructed chimeras comprising the core to NS-2 region of the related genotype 2a clone, J6, and the nonstructural regions from JFH-1. This chimera grew very well in Huh-7.5 cells, spreading more rapidly and reaching higher infectivity titers ( $10^5$ ) than JFH-1. Serial passage was achieved without the loss of infectivity. Further, it was shown that virus replication was inhibited by interferon and by protease and polymerase inhibitors, demonstrating the value of this culture system for drug testing. Zhong,<sup>118</sup> in the Chisari lab, took this a step further by deriving an "interferon-cured" cell line designated Huh 7.5.1 that allowed very efficient release of virions with sustained infectivity titers of  $10^4$  to  $10^5$  IU/mL, about 50-fold higher than in the original Wakita experiments. It is postulated that a defect in the RIG-1 pathway of innate immunity may be the reason these cells are so permissive.

In sum, these three landmark studies published in 2005 demonstrated that 1) HCV virions could replicate in human hepatoma cell lines, could be serially and efficiently passaged, and that particles produced in vitro could transmit infection to chimpanzees at reasonably high titer; 2) the HCV virion is 50-60 nm in diameter, as previously predicted, but now directly visualized; 3) infectivity could be blocked by monoclonal antibodies to E2 and by patient sera, confirming that neutralizing antibodies to HCV exist and that E2 contains a key neutralizing epitope; 4) CD81, a membrane tetraspanin, is a critical component of the HCV entry pathway; and 5) infectivity could be blocked by interferon and protease/helicase inhibitors, demonstrating the suitability of this system for evaluation of candidate antiviral agents.

Because most HCV infections are genotype 1, it was critical to establish a culture system for this dominant genotype. In 2006, Yi and Lemon<sup>119</sup> produced adaptive mutations in the nonstructural regions of the prototype genotype 1 strain, H77, and transfected this adapted genome (H77-S) into Huh 7.5 cells. Although infectivity titers with H77-S were two logs lower than with JFH-1, the system is viable and now allows study of genotypic differences in an in-vitro system. Sera from genotype-1a-infected individuals neutralized H-77S virus, but could not neutralize genotype 2a virus, a finding that has implications for the development of therapeutic hyperimmune globulins and HCV vaccines.

### Mechanisms of Viral Persistence

#### Viral Quasispecies

The most striking feature of HCV is its ability to persist in the host. Measurements of HCV RNA in serum and liver suggest that the prevalence of persistent infection is higher than the

prevalence of chronic hepatitis and may be in the range of 75% to 85%. The mechanism of persistence appears to involve the ability of the virus to mutate rapidly under immune pressure and to exist simultaneously as a series of related but immunologically distinct variants, any one of which can become the predominant strain when a coexistent strain comes under immune pressure. This coexistence of multiple genetic variants has been termed "quasispecies." It provides an efficient and rapid mechanism for the virus to escape the host immune response. Although mutations occur throughout the HCV genome, most occur in a relatively short hypervariable segment of the envelope (E2) domain. This hypervariability suggests that this region contains important target epitopes that are under intense immune attack. Among HCV isolates, the average mutation rate is  $10^{-3}$  to  $10^{-4}$  base substitutions per genome site per year.<sup>120</sup> This high rate of mutation, reflected in the quasispecies nature of the virus and in the ability of HCV to rapidly evolve escape mutants, appears to be the primary mechanism underlying the absence of effective neutralization and the development of persistent infection. A study by Farci et al<sup>121</sup> showed that early in HCV infection, patients who ultimately recover have a decreased degree of viral diversity after the appearance of anti-HCV. In these persons, antibody or coexistent cell-mediated immune responses seem to contain viral replication and quasispecies formation such that the virus becomes increasingly homogeneous and is eventually cleared. In contrast, in most patients, the development of antibody appears to drive viral diversity until the population becomes so heterogeneous that it cannot be fully contained by the immune system.

Hepatitis C virus has evolved to become an efficient machine for survival. In this survival scheme, a master strain is accompanied by a series of subservient strains that under immune pressure can themselves become the master. Defective particles are formed that can protect replicative particles, and a low level of replication can allow the virus to both hide from the host and protect its environment by inducing disease so indolent that the cells that nurture the virus are well maintained or only incrementally destroyed. These efficient mechanisms for self-survival have allowed HCV to chronically infect approximately 1% of the world's population.

### Neutralizing Antibody and Cell-Mediated Immunity

There is little evidence that HCV is directly cytopathic. It is generally believed that liver damage is the result of the host's immune response, including CTLs, cytokines, and apoptotic factors. The immune response to HCV infection is brisk and yet generally ineffective in viral clearance. High titers of antibody coexist with high levels of virus. Antibodies broadly directed across the entire genome rarely have neutralizing potential. Those directed at the critical neutralizing epitopes in the viral envelope more often tend to drive viral diversity than to eradicate the agent. Nonetheless, neutralizing antibodies to HCV have been found in mixing experiments in which chimpanzee infectivity was used as the endpoint. These neutralizing responses, however, have been shown to be transient and highly

strain-specific.<sup>122</sup> Recently, pseudoviruses, consisting of a retroviral core, HCV envelope proteins and a green fluorescence protein marker, have been developed and allow for in-vitro measurement of neutralizing antibodies to HCV.<sup>123</sup> In this system, pseudoviruses infect an HCV susceptible hepatoma cell line (Huh-7) and infected cells fluoresce green. By premixing the pseudoviruses with serum suspected to contain antibody, neutralization can be measured by diminution of green fluorescence compared to controls. This system has shown that neutralizing antibodies are formed in HCV infection, but that they appear late in the acute infection and probably play a minimal role in viral clearance.<sup>124</sup> Paradoxically, neutralizing antibodies increase over time during chronic infection and also become more broadly reactive against multiple genotypes<sup>125</sup>; this broad, cross-reactive neutralization has now been demonstrated in virus culture assays as well as in the pseudovirus system.<sup>126</sup> It is presently unclear whether these neutralizing antibodies play a role in containing chronic infection even though they are incapable of clearing the virus. It is also unknown whether these late-developing neutralizing antibodies can be harvested to produce a clinically effective HCV-specific hyperimmune globulin. A protective HCV immune globulin would be a major weapon in preventing infection of transplanted HCV-naïve livers; reinfection of transplanted livers, presumably from extrahepatic HCV reservoirs, is now universal.

An expanding array of assays to measure in-vitro lymphoproliferative and cytotoxic (CTL) responses, including enzyme-linked immunospot (ELISPOT) assays, and tetramer assays, have elucidated the cellular immune responses to HCV infection.<sup>127</sup> It has been found that cell-mediated immune responses occur early (1 to 2 weeks) after the onset of infection. These initial responses involve innate immunity, primarily effected by CD56+ NK cells. Although the liver is particularly rich in NK cells, HCV has evolved mechanisms to bypass innate immunity. It is now clear that specific HCV proteins, particularly core and NS3, directly inhibit critical steps in the transcription cascade of the innate immune response, blocking upregulation of interferon regulatory factors and the generation of natural interferons.<sup>128</sup> This impaired innate immune response allows the virus to gain an early foothold such that HCV reaches very high levels of replication before generation of the adaptive immune response, forcing the cellular immune system to combat a massively expanding and rapidly evolving viral antagonist.

Results suggest that the Class II major histocompatibility complex restricted helper T-cell ( $T_H$ ) response is particularly important in determining the outcome of acute HCV infection.<sup>129</sup> Patients with acute, self-limited hepatitis C have been shown to mount an early, vigorous, and multispecific  $T_H$  response that can be measured in the peripheral blood. In contrast, if this  $T_H$  response is weak or poorly sustained, persistent infection and chronic hepatitis ensue. It appears that in most patients with HCV infection, the  $T_H$  response is blunted. In addition to the early stimulation of  $T_H$  responses, there is a corresponding HCV-specific CD8+ CTL response that is probably driven by cytokines (IFN, IL-2) released by  $T_H$ 1 lymphocytes.



Early, vigorous, and multispecific CTL responses have been associated with viral clearance and recovery from HCV infection. Conversely, in patients with persistent HCV infection, CTL responses are weak and targeted against fewer HCV epitopes.<sup>129</sup> Although patients with chronic HCV infection have blunted CTL responses to HCV, they have normal CTL responses to other viruses such as influenza and Epstein-Barr virus (EBV). Hence, there is no generalized immunodeficiency state, but rather a specific tolerance to HCV. The net effect is that the adaptive cell-mediated immune response appears to be blunted or exhausted in those destined to become chronic carriers. It has been debated whether the exhaustion of T cells is the result of, or the cause of, viral persistence, but recent data<sup>130</sup> have demonstrated that diminished HCV-specific T-cell responses occur early in HCV infection, suggesting that the failure of this critical immune response is central and causal to viral persistence.

Thus, several mechanisms are at play in the development of persistent HCV infection including the high mutability of the virus, inhibition of innate immunity induced by viral proteins, and the blunting or exhaustion of HCV-specific adaptive T-cell responses. Further, although the total number of virions produced is large, the number of viruses per cell is small. This small intracellular viral burden may decrease the display of viral antigens on the cell surface, blunt the lymphoid immune response, and protect the cell from immune destruction. There is little evidence that HCV is directly cytopathic, so it is probable that CTL and other immune responses while attempting to contain the virus also cause liver cell damage through a variety of mechanisms, including Fas-ligand apoptosis, cytokine-induced injury (including that from tumor necrosis factor  $\alpha$ ), and perforin-induced injury.<sup>127</sup> It has been shown that Fas is upregulated on HCV-infected hepatocytes and on uninfected bystander cells and that Fas ligand is expressed on activated liver-infiltrating T cells.<sup>127</sup> Such apoptotic cell death may account for liver cell destruction that is sometimes out of proportion to the number of cells actually infected. In contrast, IFN- $\gamma$  produced by CTLs can result in intrahepatic viral clearance without accompanying liver cell injury. The T-cell-mediated attack on the HCV-infected liver may lead to sufficient inflammation and fibrotic repair that cirrhosis develops. However, in most patients with HCV infection, an equilibrium develops in which ongoing viral replication and T-cell- and cytokine-mediated cell destruction are balanced by controlled hepatic regeneration such that chronic hepatitis develops without progressive fibrosis and without impairment of liver function.

## Epidemiology

Although HCV was discovered through investigations of transfusion-transmitted hepatitis, transfusion was never the primary source of transmission and currently is only a minor mechanism for dissemination of this agent. Nonetheless, therapeutic blood transfusion is the prototype for parenteral (percutaneous) transmission of viral agents. Such percutaneous spread is defined by the intravenous infusion of contaminated blood

components or drugs or by the shared use of contaminated needles or other sharp instruments. Studies of HCV-infected blood donors showed that 27% were infected as the result of blood transfusion in the pretesting era before 1990, whereas 42% were infected by intravenous drug use (IVDU) with shared needles.<sup>131</sup> It is noteworthy that none of the blood donors in this study were drug addicts, but rather persons who experimented with drugs in their youth and generally for intervals of less than 5 years. Sharing of needles was almost universal. It is also noteworthy that there was an independent cohort who snorted cocaine and repeatedly denied IVDU. While the veracity of this denial cannot be proved, the statistical association of cocaine snorting with HCV infection is exceedingly high and substantiated in a multivariate analysis.<sup>131</sup> Further, approximately 25% of cocaine snorters provided a history of experiencing nosebleeds while snorting or observing nosebleeds in those with whom they shared snorting devices, thus providing a rationale for blood transmission by this covert route. Other routes of overt or covert blood transmission include improperly sterilized, reusable needles and dental instruments, particularly in developing nations, but also in the developed world in the decades before 1970. Although it is now difficult to prove, tattooing with shared needles or inks undoubtedly contributed to viral spread as did ritual scarification practices in native populations. Although rare transmissions have been traced to chronically infected health-care workers, the main health-care burden has been the reutilization of unsterilized needles and contaminated instruments, including early dialysis machines and inadequately sterilized diagnostic instruments such as colonoscopes and bronchoscopes. It is important to keep in mind that HCV infection requires only one exposure to result in a lifelong infection and that most of those now found to be HCV-infected were actually exposed 20 to 50 years ago when health care and transfusion practice were markedly different than they are today. Thus, our current HCV-related disease burden is primarily the residual of past parenteral spread that, except for IVDU, essentially has been interdicted in the developed world. IVDU, however, remains unchecked and has been the primary source of HCV infection since proliferation of the drug culture in the 1960s. It is conceivable that if the practice of shared-needle IVDU was eradicated, HCV infection would ultimately disappear among populations in developed countries.

Although parenteral routes are the predominant modes of HCV transmission, a parenteral source cannot be identified in up to one-third of infected individuals. This does not rule out covert parenteral exposures, but has raised the possibility of nonparenteral modes of acquisition. The CDC has conducted comprehensive epidemiologic studies of community-acquired HCV infection in four selected (sentinel) counties in the United States.<sup>132</sup> The sentinel counties studies initially revealed that among patients with clinically overt NANB hepatitis, only 15% gave a history of blood transfusion, 25% used intravenous drugs, fewer than 10% were in health-related fields or had a presumed sexual or household contact, and more than 50% had no known route of exposure to this agent. More recent evaluations<sup>133</sup> have

shown that fewer than 1% of cases of community-acquired hepatitis are related to transfusion, further demonstrating the effectiveness of donor screening measures implemented since 1990. The precise routes of nonparenteral transmission of hepatitis C remain an enigma even in the face of sensitive serologic and molecular assays for HCV. There is evidence that HCV, like HBV and HIV, has a sexual and perinatal, as well as parenteral, route of transmission. However, HCV is much less efficiently spread by these nonparenteral routes than are HBV and HIV. The CDC has implicated sexual transmission in up to 10% of acute, clinically apparent hepatitis C cases based on the absence of an identified parenteral source and on a history of having had sex with more than two sexual partners in the preceding 6 months or having had sex with a prostitute or having a recent history of a sexually transmitted disease. In contrast, the sexual partners of patients with chronic HCV infection are very rarely infected despite years of unprotected sex.<sup>131</sup> This paradox might be explained by the fact that HCV may be more transmissible early in HCV infection when viral titers are higher and when the virus is more likely to exist as free virions rather than complexed to antibody. In the final analysis, it is probable that HCV can be spread sexually, but that such spread is very inefficient and more likely to occur early in the course of infection. Because sexual transmission is rare, particularly during chronic infection, there is no mandate that committed, monogamous partners should routinely use condoms or other protective devices; rather, the probabilities and potential risks should be presented to affected couples and the decision for condom use left to their discretion. Perinatal transmission of HCV is also inefficient, but has been documented to occur in about 5% of cases where the mother is HCV RNA positive at the time of delivery.

## Clinical Spectrum of Hepatitis C Virus Infection

### Acute Manifestations

Only 25% of acute hepatitis C cases present with jaundice or substantial clinical symptoms; subclinical cases generally remain undetected until uncovered by the incidental finding of elevated ALT levels or anti-HCV during the course of blood donation or general medical/surgical evaluation. Long-term observation in prospective studies has revealed that asymptomatic acute hepatitis has the same propensity to evolve into chronic liver disease, including cirrhosis, as do the more overt cases. Thus, silent TAH may have major clinical significance. Although patients with HCV rarely have sufficient symptoms to seek medical care, individual cases can be acutely severe and even fulminant. However, fulminant hepatitis is an exceedingly rare complication of hepatitis C.

Three major patterns of elevation of ALT level have been observed in hepatitis C—monophasic, polyphasic (fluctuating), and plateau.<sup>134</sup> The monophasic pattern involves a single acute phase peak followed by complete normalization of ALT level. The polyphasic pattern is the most characteristic and includes dramatic fluctuations of ALT level over brief intervals. Over time, the amplitude of the fluctuation diminishes, but the periodicity persists into chronicity. In the plateau pattern, a relatively constant and generally low-level ALT abnormality is observed in the

acute and chronic phases of the disease. Such patients often have biopsy evidence of chronic liver disease early in the posttransfusion course. These patterns have prognostic implications. In one study,<sup>134</sup> 42% of patients with a monophasic peak progressed to chronic hepatitis, compared with 87% of patients with the fluctuating pattern and 95% of patients with the plateau pattern. Levels of ALT occasionally normalize for prolonged intervals that suggest recovery, but are followed months to years later by secondary elevations that indicate the presence of chronic liver disease. This prolonged normalization of ALT level followed by recrudescence makes it difficult to ascertain when, or if, biochemical recovery from HCV infection has occurred. The best current assessment of recovery from HCV infection is serial measurement of HCV RNA. Three negative determinations of HCV RNA over the course of 3 to 6 months generally connote sustained clearance of the virus and full recovery from the infection.

### Chronic Sequelae

The most important component of HCV infection is its ability to evolve into chronic hepatitis, cirrhosis, and more rarely, HCC. Long-term follow-up evaluation of transfusion-associated cases indicates that at least 50% of patients contract chronic hepatitis as evidenced by elevations in ALT level that persist longer than one year. More recent data indicate that chronic hepatitis may occur in nearly 70% of cases. Similar frequencies of chronic hepatitis have been documented among community-acquired cases of hepatitis C.

In eight early studies, 339 patients with transfusion-associated NANB hepatitis underwent prospective observation; 47% had biochemical evidence of chronic hepatitis.<sup>81</sup> Of 102 patients who underwent biopsy, 41% had chronic active hepatitis and 20% had cirrhosis; five of the 20 patients with cirrhosis died of liver disease. Subsequent studies in which serial liver biopsies were performed provided unequivocal documentation of the progression of histologic abnormalities from acute to chronic hepatitis to cirrhosis.<sup>135-138</sup> In summary, numerous studies of transfusion recipients have revealed the following: 1) at least 50% of patients with acute hepatitis C eventually have biochemical evidence of chronic hepatitis; 2) approximately 20% of patients with chronic hepatitis C have biopsy evidence of cirrhosis; 3) progression from acute hepatitis to cirrhosis can be documented histologically and generally evolves slowly over the course of decades but sometimes can occur more rapidly, the mean time to the development of cirrhosis being approximately 20 years; 4) the progression to cirrhosis is enhanced in patients with HCV infection who abuse alcohol—the cumulative effect of alcohol and HCV infection is more than additive, and the risk of cirrhosis may be 15-fold higher among persons who abuse alcohol than among persons with HCV infection alone<sup>139</sup>; and 5) when HCC occurs, it is almost always in patients with established cirrhosis—the risk of development of HCC among patients with cirrhosis has been estimated to be 1.7% per year.<sup>140</sup>

The foregoing considerations allow a schematic depicting the typical course of acute resolving and chronic hepatitis C (Fig 46-4). The essential features of viral replication, humoral immune responses, hepatocellular inflammation, and clinical

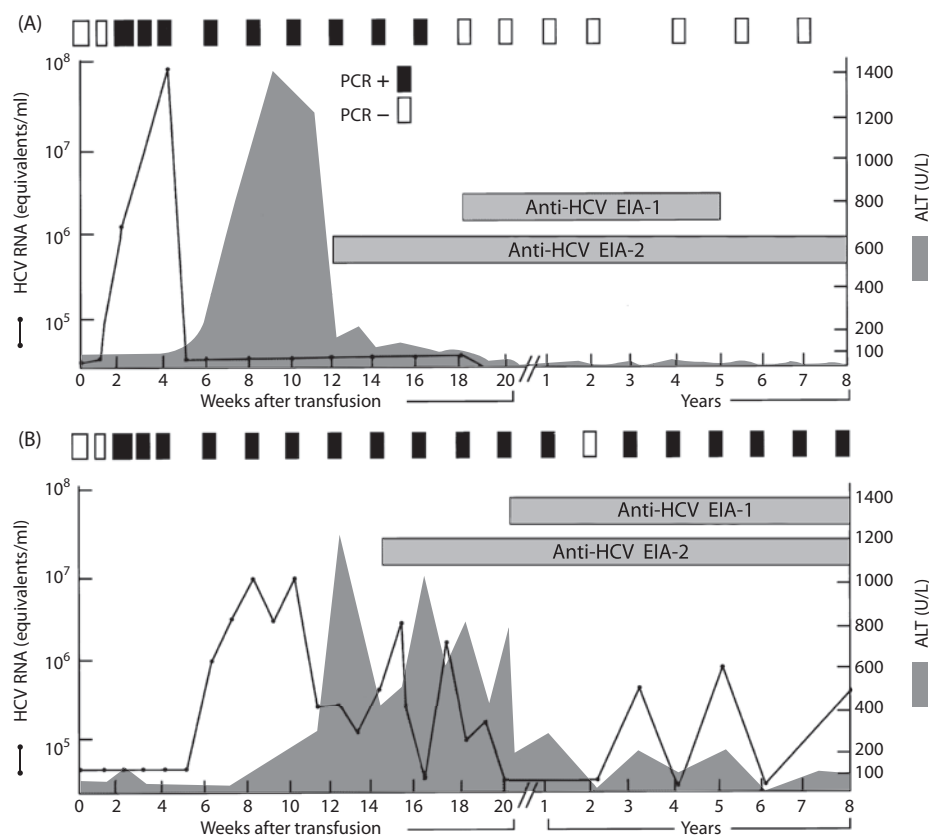


**Figure 46-4.** Biochemical, serologic, and molecular biologic profile of acute and chronic transfusion-associated hepatitis C virus (HCV) infection. Acute, resolving hepatitis C is shown in (A) and chronic hepatitis C in (B). Resolving disease cannot be differentiated from progressive disease by the time of onset of detectable HCV RNA by means of polymerase chain reaction (PCR), the magnitude of HCV RNA elevation as measured by means of branched DNA assay, the interval to the first elevation in the level of alanine aminotransferase (ALT), the magnitude of ALT elevation in the acute phase, or the interval between exposure and the first appearance of antibody. Progression to chronic disease cannot be predicted in the acute phase, and the only distinguishing features in these patterns are the persistence of ALT elevation and the persistence of HCV RNA in persons with chronic hepatitis C. The acute, resolving pattern (A) occurs in 10% to 15% of patients with transfusion-associated hepatitis C and the chronic pattern (B) in 85% to 90%. Note: 1) HCV RNA is detectable soon after exposure. Here the PCR results was positive 2 weeks after exposure, but it can become positive even sooner. 2) HCV RNA may be detected by means of branched DNA assay coincident with PCR reactivity, but the reaction may be delayed, as shown here. 3) The major

peak of viral replication (assessed with HCV RNA level) occurs before the first increase in ALT level and before any clinical or biochemical evidence of hepatitis; it is presumed that persons might be most infectious in this interval before the acute phase. 4) In acute resolving infection, HCV RNA levels increase rapidly before the decline in serum ALT level. 5) In chronic infection, HCV RNA level diminishes and can remain low, fluctuate, or become undetectable; HCV RNA levels sometimes show a periodicity that parallels the fluctuations in ALT level; in (B) the level of HCV RNA increases a short time before ALT level does and decreases before the decline in ALT. 6) Second-generation anti-HCV assays shorten the seronegative window in HCV infection much more than do first-generation assays; nonetheless, anti-HCV was not detectable for 12 to 15 weeks after exposure and for 6 to 7 weeks after the first significant rise in ALT level. Antibody to HCV (detected with second-generation assays) almost always persists in chronic cases and generally persists in acute resolving cases. Antibodies detected with the first-generation assay (anti-C100, anti-5-1-1) generally disappear in resolving cases. (Used with permission from Alter.<sup>105</sup>)

outcome are described earlier and in the legend to Fig 46-4. These are representative diagrams of classic cases—there is considerable variability in these patterns from case to case. The most consistent features are that viral replication can be detected soon after exposure, that peak viremia occurs in the acute or preacute phase in patients who recover and in those who have chronic hepatitis, and that progression to chronic hepatitis cannot be predicted by the time of onset or the peak level of viremia.

Untoward occurrences among recipients of standard blood transfusion are exaggerated in recipients of clotting factor

concentrates. Before recent inactivation procedures were developed, such concentrates, derived from massive pools of paid blood donors, were invariably contaminated with HBV and HCV. Overall, more than 50% of patients with hemophilia have ALT elevations and serologic results that suggest chronic hepatitis C.<sup>141</sup> That proportion increases to 80% among patients with severe hemophilia who received clotting factor concentrates before virus inactivation.<sup>142</sup> In a landmark multicenter study,<sup>142</sup> liver biopsy specimens from 155 patients with hemophilia and chronic elevations in ALT level revealed cirrhosis in 15% and

severe chronic active hepatitis, likely to progress to cirrhosis, in an additional 7%. Similar severe outcomes were found in a subsequent prospective study in England<sup>143</sup> wherein 71% of 79 patients with hemophilia had persistent abnormalities in ALT level, 25% of the 34 patients who underwent biopsy had cirrhosis, and 12% had radiographic evidence of esophageal varices. One patient died of complications of portal hypertension. Before AIDS, chronic liver disease accounted for 5% to 11% of deaths in patients with hemophilia. The advent of AIDS has further complicated the course of hepatitis C in this population. It is now evident that HIV infection accelerates the course and increases the severity of coexistent HCV infection.<sup>144</sup> Now that patients with HIV infection have long survival because of highly active antiretroviral therapy, severe liver disease is emerging as a major cause of morbidity and mortality among patients with HCV and HIV coinfection.

In Japan, where hepatitis C is common and where a similar progression from acute to chronic hepatitis has been well documented, it has been possible to examine the role of blood transfusion in the development of chronic liver disease. Kiyosawa et al<sup>145</sup> found that among patients with chronic hepatitis and cirrhosis, presumably related to hepatitis C (then designated NANB hepatitis), 44% and 38% of patients, respectively, had a history of previous transfusion. In HCV-related chronic liver disease, a history of transfusion was 6 to 12 times more frequent than in HBV-related disease. This finding suggested a causal relation in the former, but less so in the latter, where the virus tends to be transmitted more readily by sexual and maternal-fetal routes. Another important observation in the study of Kiyosawa et al was the long interval between transfusion and the first clinical manifestations of chronic liver disease. The mean interval from transfusion to the clinical recognition of chronic hepatitis was 11.3 years; to the development of overt cirrhosis, 21.2 years; and to the development of clinically apparent HCC, 29.0 years.<sup>145</sup> These long intervals may account for the apparent disparity between the number of expected cases of transfusion-associated cirrhosis and the number of cases actually detected; because patients who undergo transfusion tend to be elderly, many die of an underlying illness before chronic liver disease becomes clinically evident. Such intercurrent deaths obscure the course of this slowly evolving disease and mask the possible effect of HCV-related chronic hepatitis and cirrhosis on younger blood recipients.

Infection with HCV can unequivocally lead to HCC.<sup>146</sup> In a composite of prospective studies, it appears that the overall incidence of HCV-associated HCC is 1% to 5%. However, once cirrhosis develops in chronic hepatitis C, the risk of developing HCC is about 4% per year. Currently, the rate of HCV-related HCC is relatively low in Western nations and relatively high in Japan. Okuda et al<sup>147</sup> conducted a study of 113 patients with nonalcoholic HCC and found that 69% of the cases of HCC were HBsAg negative and presumably related to HCV infection, as subsequently documented by serologic testing.<sup>148</sup> The disparity between the frequency of HCV-related HCC in the United States and Japan probably reflects the duration of infection in the

respective populations. Calculations using the molecular clock methodology suggest that the major spread of HCV in Japan preceded that in the United States by 30 to 40 years.<sup>149</sup> Hence, the infected population in Japan is decades older and their duration of infection places them at increased risk of HCC evolution. It is anticipated that as the duration of infection in Western populations increases, the rates of HCC will increase concomitantly and hence that many more cases will be observed in the next 2 to 3 decades unless chronic HCV carriers can be successfully treated in the interim.

The long-term consequences of TAH were comprehensively studied by Seeff et al.<sup>150</sup> Patients who had TAH in five major prospective studies conducted in the 1970s were traced after a mean interval of 18 years and prospectively evaluated for evidence of chronic hepatitis. Over this interval, there was no increase in overall mortality among patients who sustained an episode of TAH compared with controls who underwent transfusion and did not have an episode of TAH. Although the mortality was not different, 35% of patients with previous episodes of hepatitis had biochemical and histologic evidence of chronic hepatitis compared to 1% of controls. The results of this study were subsequently updated to a mean follow-up period of 23 years.<sup>151</sup> Overall mortality was similar among patients with hepatitis and control subjects without hepatitis, but patients with hepatitis had a small, but significant increase in liver-related mortality (4.1% vs 1.3%;  $p = 0.05$ ). Among 103 patients with positive results for anti-HCV in 1974, 76% were still anti-HCV positive and HCV RNA-positive approximately 20 years later. An additional 17% had specific anti-HCV but no longer had viremia, suggesting they had spontaneously cleared the infection. A surprising finding was that 7% of patients had no residual markers of HCV infection, suggesting they not only had cleared the virus, but also had lost anti-HCV. Hence, prevalence estimations based on serologic status may underestimate the extent of HCV infection in the population. More important, this study showed that the spontaneous clearance rate of HCV infection may exceed the commonly accepted estimate of 15% and actually be between 20% and 25%. In a separate study of longer duration, Seeff et al<sup>152</sup> found that only 1 of 17 persons with chronic HCV infection had died of liver disease in the nearly 50 years after their HCV infection was first documented.

Milder outcomes of HCV infection have been observed in studies of asymptomatic blood donors found to be anti-HCV positive at the time of donation. In an NIH study,<sup>131</sup> 15% of donors appeared to have spontaneously cleared HCV infection on the basis of the presence of RIBA-confirmed anti-HCV and repeatedly negative determinations for HCV RNA. Among those with persistent HCV infection, the peak ALT level exceeded two times the upper limit of normal in only 16%, and clinical symptoms were minimal. Liver biopsy was performed on 94 patients. No patients had severe inflammatory changes, 13% had stage 3 or 4 fibrosis, and only 2% had cirrhosis after an average duration of infection of 19 years on the basis of a defined parenteral exposure. Repeated liver biopsies were performed 5 years later on

60 of these patients. Fibrosis progression over that interval was minimal; no new cases of cirrhosis were found even when the follow-up was extended to a mean of 24 years.<sup>153</sup>

Three additional long-term outcome studies also suggest relatively mild outcomes in chronic HCV infection. Kenny-Walsh<sup>154</sup> performed a 17 year follow-up study of 376 women with HCV infection from a contaminated lot of Rh Immune Globulin (RhIG). Liver biopsies were performed on 363 patients a mean of 17 years after exposure; only 4% had moderate to severe grades of inflammation. Periportal fibrosis (stage 1) was found in 34% of patients; bridging fibrosis (stage 3) in 15% and cirrhosis in only 2%; 49% had no fibrosis. Two of seven patients with cirrhosis also had a history of alcohol abuse. A similar study in Germany<sup>155</sup> enrolled 152 women with HCV infection traced to contaminated lots of RhIG and found that none had cirrhosis or severe fibrosis after a mean follow-up period of 15 years. Further supporting mild outcomes of HCV infection, a 20-year follow-up study<sup>156</sup> of children who were infected at the time of heart surgery revealed that 45% had spontaneously recovered based on the findings of RIBA-confirmed anti-HCV and persistently negative assays for HCV RNA. Among the children with viremia who underwent biopsy, only two of 17 had portal fibrosis and only one had cirrhosis 20 years after exposure. The one patient with cirrhosis also had chronic HBV infection.

There is a paradox in the course of HCV infection. If one studies patients who already have evidence of severe liver disease, it is evident that HCV is responsible for the majority of cases. From this perspective, HCV projects as a frequent cause of cirrhosis, end-stage liver disease, and HCC. On the other hand, if one prospectively evaluates cohorts of persons with HCV infection, a more balanced picture emerges. First, as indicated above, a minimum of 15%, and possibly as high as 25% of infected adults spontaneously recover from HCV infection, usually within the first year after onset. The rate of recovery among children with HCV infection is even higher, reaching 45% in a retrospective-prospective study conducted in Germany.<sup>156</sup> It is now also apparent that in the absence of alcohol abuse or coexistent HIV infection, chronic hepatitis C is generally an indolent process in which severe outcomes may never occur or may take decades to evolve. Most adults who undergo transfusion die of the disease for which they were transfused or of an intercurrent illness before the potentially fatal complications of chronic hepatitis C become manifest. Nonetheless, over the span of 20 or more years, 20% to 30% of patients with HCV infection may have histologic evidence of cirrhosis that can ultimately lead to liver failure and the need for liver transplantation. That the majority of patients with HCV infection spontaneously recover or have a nonprogressive form of chronic liver disease gives hope to the individual patient; however, this does not diminish the global impact of HCV infection engendered by the sheer magnitude of the population that is infected. On a global scale, the absolute number that face life-threatening events related to HCV is staggering even if the proportion that encounter these events is encouragingly small.

## Treatment

The last decade has seen marked advances in the treatment of hepatitis C and new classes of drugs are in the pipeline. The foundation of therapy since the inception of treatment has been IFN- $\alpha$ , but the introduction of combination therapy with ribavirin and subsequently, the use of pegylated interferon have been the innovations that have increased sustained efficacy from approximately 10% in the early 1990s to near 50% at present.<sup>157</sup> The efficacy of treatment is assessed by the sustained loss of HCV RNA. During treatment, four patterns of HCV RNA response are observed: 1) **Non-response** wherein HCV RNA levels do not fall or are only minimally reduced during therapy; 2) **Breakthrough** wherein a good initial response is followed by a return of HCV RNA to original levels despite continued therapy; 3) **Relapse** wherein HCV RNA becomes undetectable during therapy, but then rebounds to initial levels soon after therapy is discontinued; the main benefit of ribavirin is its ability to prevent this virologic relapse; 4) **Sustained virologic response (SVR)** wherein HCV RNA becomes undetectable during therapy and remains undetectable at least 6 months after the cessation of therapy. Considerable evidence now shows that a sustained virologic response is tantamount to “cure” because long-term follow-up studies have not shown recrudescence of infection beyond 6 months after therapy is terminated. Serial measurements can be used early after initiation of therapy to assess the likelihood of response. Those who have a more rapid early response are more likely to have a sustained response.<sup>157</sup> There are several parameters that predict treatment outcome, but none are absolute. In general, higher response rates are seen in females, in those less than age 40, in those of European ancestry compared to those of African ancestry, in the nonobese and in those with viral loads less than 2 million copies/mL. In addition, persons with severe liver disease, particularly cirrhosis, and those with immunodeficiency, particularly HIV infection with CD4 counts  $<200 \text{ mm}^3$ , are less likely to respond. However, none of these parameters are a contraindication to treatment and their value lies in predicting the probability of response. The main determinant of virologic response is the viral genotype; SVR in persons who test positive for genotype 1 or 4 approximates 45% to 50% with combined IFN- $\alpha$  and ribavirin, whereas patients with genotype 2 or 3 infection have an approximate 80% sustained virologic response. In addition, patients with genotype 2 or 3 infection have to be treated for only 6 months compared to a 1-year course for those with genotypes 1 and 4.<sup>157</sup> Hence, it is essential to obtain a viral genotype before initiating therapy. Another important treatment guideline is that treatment can be stopped after 3 months in persons whose viral load has failed to become undetectable by that time; the continued presence of virus in the peripheral blood 3 months after initiation of therapy is highly predictive of treatment failure.

In summary, the combined use of pegylated IFN- $\alpha$  (180  $\mu\text{g}$ /wk, subcutaneously) and weight-based dosing of ribavirin (800–1200 mg/day, orally) will result in an approximate 50% SVR for genotypes 1 and 4 and an approximate 80% SVR for genotypes 2 and 3. These treatments have multiple side effects that are

often poorly tolerated. Interferon can cause flu-like symptoms, marked fatigue, mood changes (particularly depression and irritability), hair loss, anorexia, nausea and vomiting, thyroid dysfunction (generally hypothyroidism) and cytopenias, particularly thrombocytopenia and leukopenia. All these side effects, except those involving the thyroid, are reversible upon cessation of therapy. Cytopenias may necessitate dose reduction or cessation of interferon therapy. The primary serious side effect of ribavirin is hemolytic anemia. This can be offset by administration of erythropoietin, but often requires dose reduction or discontinuation of this arm of the therapy. The mechanisms of interferon's and ribavirin's antiviral effects are diverse and not completely understood, but they are general antiviral agents and not HCV-specific therapies. Under development are several drugs that are specifically directed to critical steps in the HCV life cycle, including inhibitors of HCV protease and helicase enzymes. Early evidence indicates that this class of virus-specific enzyme inhibitors are very potent and can decrease viral loads rapidly.<sup>158</sup> However, as with similar inhibitors for HBV and HIV, resistance develops rapidly and hence these drugs will not be given singly, but rather in combination with interferon, interferon plus ribavirin, or with each other. It is probable that interferon will remain the backbone of HCV therapy and that these new designer drugs will be additive rather than replacements. It is anticipated that under these new combination regimens, treatment efficacy will increase significantly, but the substantial side effects of interferon will remain and side effects of the new agents will be added. It is hoped that the anticipated additive efficacy of these new therapies will offset their side effects and resistance patterns, especially because the drug cocktails may need to be given for only 6 months. Many other therapeutic approaches, including small interfering RNAs, are under investigation and the ability to study new agents has been enhanced, not only by increased knowledge of the structure of the virus, but also by the recent availability of in-vitro culture systems<sup>116-119</sup> and the chimeric severe combined immunodeficiency (SCID) mouse model that harbors human hepatocytes.<sup>159</sup> Last, the potential for liver transplantation is increasingly available for those who fail antiviral therapy and progress to end-stage liver disease. Although the transplanted liver is universally reinfected, the 5-year survival for liver transplantation now approximates 70%.<sup>160</sup> Nonetheless, in a subset of transplant recipients, recurrent hepatitis C is severe and cirrhosis may develop in as little as 3 to 5 years.<sup>161,162</sup> The main impediment to liver transplantation is not graft or patient survival, but rather cost and the inability to procure a sufficient number of viable livers for the very large population that has developed, or subsequently will develop, HCV-related end-stage liver disease that is not amenable to current drug therapies.

### Non-A, Non-B, Non-C Hepatitis

Within the spectrum of TAH, cases were identified that met the biochemical and exclusionary definition of NANB hepatitis,

but were subsequently shown to be negative for serologic and molecular markers of HAV, HBV, and HCV infection. Such cases have been termed non-A, non-B, non-C hepatitis and have been assumed to be the consequence of another, as yet undiscovered, human hepatitis virus. However, such cases could also be the result of a known hepatitis agent present at levels below the detection limit of current assays, or be of nonviral origin. After more than a decade of intensive investigation, and despite many published and unpublished claims of discovery, a non-A, non-B, non-C human hepatitis agent has yet to be conclusively identified.

Evidence for the existence of more than one NANB hepatitis agent initially derived from the clinical observation of more than one episode of HBV- and HAV-negative hepatitis occurring in the same patient<sup>163</sup> and the observation that some episodes of NANB hepatitis among persons with hemophilia occurred after an unusually short incubation period.<sup>164</sup> Results of early chimpanzee cross-challenge studies further supported the existence of two NANB agents.<sup>165</sup> One of these agents proved to be HCV, and the other is still undefined. Confounding the issue of whether there are additional NANB agents is the observation that both humans and chimpanzees can sustain more than one episode of HCV infection.<sup>166</sup> Thus, what appeared to be two episodes of NANB hepatitis may instead have been two distinct episodes of hepatitis C. Nonetheless, 10% to 30% of cases of chronic NANB hepatitis cannot be accounted for by HCV infection, and most cases of fulminant hepatitis and hepatitis-associated aplastic anemia appear to be unrelated to hepatitis viruses A, B, or C, suggesting the existence of an additional agent of hepatitis in humans.

Encouraged by the blind cloning of HCV, investigators have used sophisticated molecular approaches to search for the agent or agents of non-A, non-B, non-C hepatitis. This has led to the sequential discovery of the GB virus<sup>167</sup> the hepatitis G virus,<sup>168</sup> the TT virus,<sup>169</sup> and the SEN virus.<sup>170</sup> None of these agents has yet been proven to be a hepatitis virus in humans, but each has been shown to be transmitted by transfusion and to result in persistent infection in some blood recipients. The GB agent derives from the blood of a surgeon (G.B.) who in the 1950s contracted acute icteric hepatitis. Inoculation of GB serum into marmosets reproducibly caused hepatitis, but controversy arose as to whether this represented transmission of a human hepatitis agent or reactivation of a latent marmoset hepatitis virus. The controversy was never resolved. Decades later, investigators at Abbott Laboratories analyzed frozen samples that had been collected during serial passages of the GB agent in the marmoset model. Using representational difference analysis to compare preinoculation and postinoculation marmoset serum,<sup>167</sup> the investigators identified unique nucleic acid sequences in the postinoculation specimens.<sup>171</sup> Sequencing of the full genome identified three distinct GB agents that were designated GBV-A, GBV-B, and GBV-C.<sup>171</sup> The genomic structure identified these agents as flaviviruses, but the agents showed little homology to HCV or other members of that family. Extensive population studies involving

patients with and without liver disease and studies of marmosets established that GBV-A was a primary marmoset agent, that GBV-C was a human virus, and that GBV-B might infect both species.

In independent investigations, Gene Labs (in collaboration with NIH) and the CDC, used sequence-independent single primer amplification to identify what was considered a novel hepatitis agent and called the hepatitis G virus.<sup>168</sup> Subsequent sequence comparisons revealed that GBV-C and HGV were essentially the same agent and represented strain variants of this new member of the *Flaviviridae* family. Although GBV-C/HGV was unequivocally found to be transmitted by transfusion, extensive clinical studies did not show a causal relation between the presence of the virus and the development of hepatitis.<sup>172</sup> The designation hepatitis G virus now appears to be a misnomer; it would be better to refer to this agent as the GB virus because that designation does not infer causality.

In 1997, Nishizawa et al<sup>169</sup> in Japan used representational difference analysis to compare pre- and posthepatitis sera from five patients with TAH. A unique viral clone was found in three of the five cases of TAH studied, and viral titers generally correlated with the ALT level. The presumed virus was designated TT virus after the initials of the index case. Biophysical characterization<sup>173</sup> suggested that TT virus was a novel, 3700 bp, nonenveloped, circular, single-stranded human DNA virus most closely related to the *Circoviridae*, a family of plant and animal viruses not previously associated with human disease.<sup>174</sup> The TT virus family is highly divergent and at least 16 genotypes have been identified.

The TT virus has been reported to replicate in the liver based on PCR detection and in-situ hybridization.<sup>175</sup> However, there were no morphologic changes in the liver cells that showed hybridization signals, and there was no correlation between the percentage of TT-virus-infected hepatocytes and the histologic activity index or its composite scores. Although the TT virus can infect liver cells, it may not cause hepatitis. Epidemiologic studies have shown that TT virus is transmitted by both parenteral and enteral routes. The original studies of TT virus suggested a relation to acute and chronic liver disease, but subsequent studies with inclusive primers that targeted conserved sequences showed an exceedingly high background prevalence (93%) and thus established that most persons with TT virus infection did not have hepatitis.<sup>176</sup>

Matsumoto et al<sup>177</sup> studied TT virus infection in the transfusion setting using the more restrictive primers used in the original publications. The background prevalence in the volunteer US donor population was 7.5%. The key finding of this NIH study was that the frequency of transfusion-associated TT virus infection was identical among patients with non-A–E hepatitis (see later) (23%) and control patients who did not have hepatitis (22%). Overall, fewer than 4% of TT virus infections were associated with hepatitis, and there was poor correlation between TT virus DNA levels and ALT levels. Hence, as had occurred in investigations of HGV, the NIH prospective series did not support a causal association between TT virus infection and

posttransfusion hepatitis. However, there are caveats to the interpretation that the TT virus lacks pathogenicity in that the agent may cause disease in only a small number of infected persons who have particular host susceptibility factors or there may be particular TT virus variants that are pathogenic while the wild-type virus is not.

Many other isolates subsequently were identified in the TT virus family, including agents designated SANBAN, YONBAN, and TLMV.<sup>178</sup> Each of these agents is phylogenetically distant from the prototype TT virus agent and shows only 50% to 60% sequence homology. Unlike HCV variants that primarily represent strain differences of 1% to 2% (quasispecies) or genotypes diverging by approximately 15%, in the TT virus family, sequence differences between variants frequently exceed 30% and sometimes exceed 50%. These differences are so great that the agents, although linked by common biophysical characteristics, may have totally different clinical spectrums and disease associations.

A virus detection program conducted by the Diasorin Corporation (Stillwater, MN) uncovered what was at the time considered a novel infectious agent and designated SEN virus for the initials of the patient in whom it was initially found. Like TT virus, SEN virus proved to be a small, nonenveloped, circular, single-stranded DNA virus.<sup>179</sup> The SEN virus represented a subfamily of very heterogeneous agents differing in nucleotide sequences by 15% to 50% from each other and by 40% to 60% from the prototype TT virus sequence. The SEN virus is now considered a member of the TT virus family in the genus *Circoviridae*.

The clinical significance of SEN virus is uncertain. Two SEN virus variants, designated SENV-D and SENV-H, have been extensively studied.<sup>179</sup> SENV-D and -H were found in 1.5% to 2% of volunteer US donors and were shown to be transfusion transmitted. They were present in 40% of 155 subjects who underwent transfusion but in only 3% of 97 control subjects who did not undergo transfusion ( $p < 0.0001$ ).<sup>179</sup> A significant relationship of infection to transfusion volume was observed, and donor-recipient linkage was established by means of sequencing. It is noteworthy that new SEN virus infections occurred in 3% of patients who did not receive transfusions. This finding suggested that, as with TT virus, there was nosocomial transmission or reactivation of latent virus.

In the NIH prospective study of TAH,<sup>179</sup> 13 cases of non-A–E hepatitis were found. One of these patients was infected with SEN virus before transfusion. Among the remaining 12 susceptible recipients, 11 (92%) became SENV-D or SENV-H positive after transfusion. There was a good, although imperfect, temporal association between the level of virus and the level of elevation of ALT value. In contrast, acute SEN virus infection was found in 24% of patients who underwent transfusion but did not have hepatitis ( $p < 0.0001$ ). Despite the strong statistical link to cases of transfusion-associated non-A-E hepatitis, it was projected that no more than 5% of cases of SEN virus infection were accompanied by biochemical evidence of hepatitis.

Further, there has been no significant association between SEN virus infection and acute liver failure or cryptogenic cases of chronic hepatitis, cirrhosis, or HCC. If SEN virus is a hepatitis agent, it causes disease in only a small number of infected individuals, the difference perhaps depending on host susceptibility, viral load, or immune responsiveness. A parallel might be drawn to viruses such as cytomegalovirus or EBV that also cause disease in only a small number of infected persons. Nonetheless, it is important to emphasize that the statistical association between SEN virus infection and TAH does not establish causality. There is currently no proof that either GB virus, TT virus, or SEN virus represents the elusive agent of non-A, non-B, non-C hepatitis.

An epidemic form of NANB hepatitis has resulted in massive outbreaks, most prominently in India, other parts of the Asian subcontinent, and South America.<sup>180,181</sup> These explosive outbreaks are waterborne and epidemiologically resemble HAV infection, but they are serologically and to some degree clinically distinct. Particles ranging from 27 to 38 nm have been associated with the epidemic form of NANB hepatitis and a specific serologic assay has been developed.<sup>182</sup> This form of hepatitis is now known to be caused by a distinct RNA virus designated hepatitis E virus (HEV) and to be classified in the family of *Caliciviruses*. Although HEV was first recognized in its epidemic form, it is endemic in many areas of the world and is probably maintained in animal hosts, particularly swine. An HEV vaccine has been developed, but not yet widely applied.

Thus, the characterized human hepatitis viruses range from A to E, including HAV (infectious), HBV (serum), HCV (former NANB), HDV ( $\delta$  agent), and HEV (enterically transmitted non-A, non-B). It is unclear at present whether a non-A, non-B, non-C transfusion-transmitted hepatitis agent exists or whether these biochemically defined cases represent cryptic (sero-silent) forms of hepatitis B or C or are of nonviral etiology. Viral discovery programs are ongoing, but their likelihood of success diminishes as the incidence of non-A, non-B, non-C hepatitis declines in the wake of enhanced blood donor screening. Clearly, the discovery of HCV and the introduction of molecular assays for HCV RNA coupled with increasingly sensitive assays for HBsAg has diminished TAH incidence to minuscule levels and proportionately reduced the clinical relevance of non-A, non-B, non-C hepatitis in this setting.

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## Summary

A marked decline in the incidence of TAH was observed in the early 1970s as commercial blood supplies were phased out and the United States adopted an all-volunteer donor system. No subsequent interdictive measure has had such dramatic effect, although additional contributions were made by the development of sensitive assays for HBsAg and by the introduction of surrogate tests for the NANB hepatitis agent. Ironically, AIDS had the next most profound effect on the incidence of hepatitis.

The development of a highly sensitive test for antibody to HIV and the intensified donor screening initiated to exclude high-risk AIDS groups served to exclude the primary source of donors transmitting viral hepatitis. The overlap between groups at risk of AIDS and groups at risk of viral hepatitis is so marked that a single exclusionary process diminishes both occurrences. As the result of these indirect measures, the incidence of TAH had declined significantly even before the introduction of anti-HCV testing. In sequential prospective studies at the NIH, the incidence of TAH had decreased from 8% to 10% to approximately 4% just before the introduction of routine anti-HCV donor screening.

Implementation of anti-HCV testing resulted in an approximate 80% reduction in TAH incidence from pretesting levels, bringing the frequency down to less than 1%. It can be calculated from the prevalence of the HCV carrier state (0.3%), the number of units of blood components transfused (15 million annually), and the infectivity of anti-HCV-positive, RIBA-positive blood (90%) that the introduction of first-generation assays initially prevented 111 transfusion-associated HCV infections per day in the United States alone, or approximately 40,000 infections per year. A subsequent analysis showed that second-generation assays would find one additional true positive donor in every 1000 tested and thus could have prevented an additional 15,000 HCV transmissions per year. A prospective study at the NIH in which the second-generation anti-HCV assay was used for donor screening showed that the risk of transfusion-transmitted hepatitis C is now exceedingly low and, indeed, approaches zero—a remarkable achievement given that the incidence of hepatitis was 33% in the 1960s and 10% to 12% in the early 1980s. Mathematical modeling after the introduction of HCV-NAT places the risk of transfusion-associated hepatitis C at one case per 1.5 to 2.0 million transfusions. Although anti-HCV screening has been a critical determinant in risk reduction, it has been only one of many interventions since 1985 that have increased blood safety. Other measures include the more judicious use of blood components and derivatives by physicians and a concerned recipient population; the increasing use of autologous blood and blood management techniques; intensive donor questioning and interdiction based on high-risk behavior; the shift to apheresis platelets; the introduction of additional infectious disease tests; bacterial testing of platelet products; and virus inactivation of clotting factor concentrates. Under development are chemical pathogen reduction procedures for plasma, platelets, and red cells. These combined sociologic, serologic, molecular, and virus inactivation measures raise the realistic potential that transfusion-associated viral hepatitis will be totally eradicated and become a historical footnote in the practice of transfusion medicine.

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## Disclaimer

The authors have disclosed no conflicts of interest.



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Ever since the advent of the global human immunodeficiency virus (HIV) epidemic, transfusion medicine has maintained a heightened awareness of the potential risk of transmitting infection through blood transfusions. This awareness has paradoxically increased despite the actual infectious risks of blood transfusion being lower than ever in developed countries. The enhanced level of safety has resulted from a series of stepwise interventions and policies in blood services throughout the world. Microbial risks were once mainly related to persistent infections such as HIV, hepatitis B virus (HBV), and hepatitis C virus (HCV), where viral carriage or latency were the key factors predisposing an agent to transmission by transfusion. More recently, several acute infections have posed a threat to blood safety when they have occurred at high incidence in certain populations. West Nile virus (WNV) in North America is a good example of this but other infections such as chikungunya and the specter of respiratory viruses such as pandemic influenza have made transfusion microbiologists widen their areas of vigilance. These issues are reflected in this chapter.

## Retroviruses

### Overview

Retroviruses are widely distributed in nature, with examples in insects, reptiles, and almost all mammals. There are four main pathogenic human retroviruses—namely, HIV-1 and HIV-2, belonging to the lentivirus group of the retrovirus family, and human T-cell lymphotropic viruses (HTLV-I and HTLV-II), belonging to the oncornavirus group. It is generally accepted that HTLV-I and HTLV-II evolved from simian T-lymphotropic retroviruses that were transmitted to humans centuries ago. HIV-1 is also thought to have derived from a simian ancestor, as simian immunodeficiency viruses are endemic in chimpanzees

in Central Africa.<sup>1</sup> HIV-2 originated from sooty mangabey monkeys in West Africa. It is thought that transmission to African natives may have occurred only over the last century.<sup>2,3</sup>

There are three groups of HIV-1, the main group (M) containing several genotypes A through K, new group (N), and the outlier group (O). Group M is widely distributed throughout the world and is the predominant cause of the AIDS pandemic. Many of the group M subtypes and most of the circulating recombinant forms have a limited geographic spread (Table 47-1). Genotype B is by far the most widespread HIV genotype in the developed world, whereas genotype C is the most common overall, accounting for 50% of HIV infections. The Democratic Republic of Congo has the largest degree of HIV-1 genetic diversity, suggesting that this geographic area is the source of the virus.<sup>4,5</sup> Groups O and N are confined to Cameroon in Central Africa. HIV-2 was originally thought to be confined to West Africa, but international travel has resulted in a wider distribution that represents 1% or less of all HIV infections.

Retroviruses are membrane-coated, single-stranded RNA viruses that require the essential enzyme, reverse transcriptase, to transcribe their viral RNA to complementary double-stranded DNA that will integrate into the host cell chromosome (Fig 47-1). The host cell enzymes aid the virus to complete its life cycle by synthesising virions that bud from the plasma membrane to infect other cells or organisms. More virologic and clinical details on retroviral infections can be accessed in the scientific literature.<sup>7-12</sup>

### Human Immunodeficiency Virus

Originally described in the early 1980s by both Montagnier and Gallo, respectively, as lymphadenopathy virus (LAV) and human T-cell lymphotropic virus type III (HTLV-III), HIV-1 (as it subsequently became known) was established as the cause of AIDS in 1984 (Fig 47-2). Shortly after infection, the virus integrates into the host cell DNA using reverse transcription. The infected individual may suffer a “glandular fever”-like “seroconversion” illness within 2 to 3 weeks of infection, then develops antibodies, and usually remains asymptomatic for many

**Table 47-1.** Human Immunodeficiency Virus Strains Found Worldwide\*

| HIV Strain    | Subclassification | Main Geographic Concentration                      | Worldwide Prevalence |
|---------------|-------------------|----------------------------------------------------|----------------------|
| HIV-1 Group M | A                 | Africa and former USSR                             | High                 |
|               | B                 | America, Western Europe, Australia                 | High                 |
|               | C                 | South and East Africa, India                       | High                 |
|               | D                 | East and West Africa                               | High                 |
|               | F                 | West Central Africa                                | Very low             |
|               | G                 | West Central Africa                                | Very low             |
|               | H                 | West Central Africa                                | Very low             |
|               | J                 | West Central Africa                                | Very low             |
|               | K                 | West Central Africa                                | Very low             |
| HIV-1 CRF     | CRF01_AE          | South East Asia                                    | High                 |
|               | CRF02_AG          | West Africa                                        | High                 |
|               | CRF03 to CRF16    | Africa, China, Thailand, Argentina, Western Europe | Very low             |
| HIV-1 URF     | Several           | Africa, South America, South East Asia             | Moderate             |
| HIV-1 Group O |                   | West Central Africa                                | Very low             |
| HIV-1 Group N |                   | West Central Africa                                | Very low             |
| HIV-2         | Sub types A and B | West Africa                                        | Moderate             |

CRF = circulating recombinant forms (component subtypes after the underscore); URF = unique recombinant forms

\*Adapted from McCutchan.<sup>3</sup>

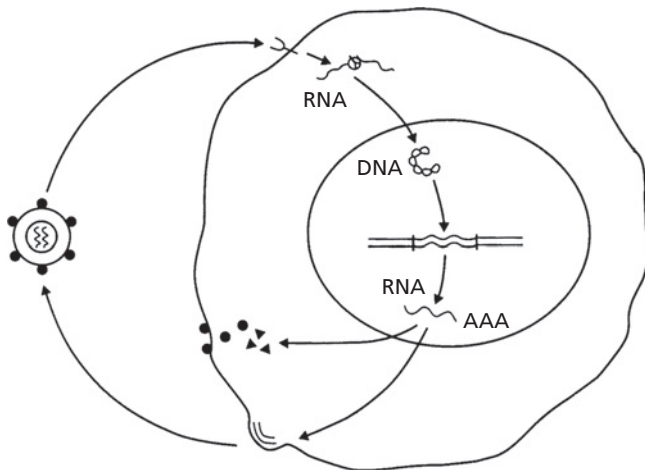


**Figure 47-1.** Simplified replication cycle of a retrovirus. The virion containing two RNA genome copies enters the cell via a specific cell surface receptor. The single-stranded RNA genome is converted into a double-stranded RNA provirus by the virion enzyme reverse transcriptase. The provirus inserts into host chromosomal DNA in the same orientation as the original virion RNA. Transcription of RNA from the integrated DNA provirus is mediated by cellular RNA polymerases, and this RNA serves both as messenger RNA for the synthesis of viral antigens and as genomic RNA, which becomes packaged into progeny virion budding from the cell surface. Used with permission from Weiss et al.<sup>6</sup>

years. However, during this asymptomatic period, the latent virus commonly reactivates and actively replicates in lymphoid tissues, macrophages, and other tissues. The number of CD4<sup>+</sup> lymphocytes gradually decreases until the infected individual

becomes increasingly prone to opportunistic infections, indicating the start of clinically apparent AIDS. In the developed world the use of combination drug therapy can prolong life expectancy, but the majority of those denied such medication will inevitably reach the terminal stage of the disease.

### Incidence and Prevalence Among Blood Donors

Since 1983 men who have had sex with other men (MSM) have been permanently excluded from donation in most countries because the HIV prevalence was high in this population. With improvements in HIV serologic tests [including the introduction of HIV nucleic acid amplification testing (NAT)], the risk reduction measures have been subject to review following pressure from this population group. Although it is expected that potential donors will answer donor questionnaires honestly, it has been obvious from counseling HIV-positive donors that a significant proportion admit to a risk factor that would have resulted in deferral.<sup>14</sup>

The number of new cases of HIV infection (incidence) among blood donors should reflect the situation within the general heterosexual population. In the United Kingdom (UK) new cases of HIV have doubled in the general population during the past 5 years. This is a cause for concern and donor statistics have also shown a general increased trend to over twice the usual number of HIV-positive donors being detected in 2002 to 2003 compared with 2000 to 2001. The rate in new donors peaked in 2005 with nine cases per 100,000; the rate in repeat donors peaked in 2002 to 2003 with 0.8 case per 100,000.<sup>15</sup> The HIV-positive donor rate

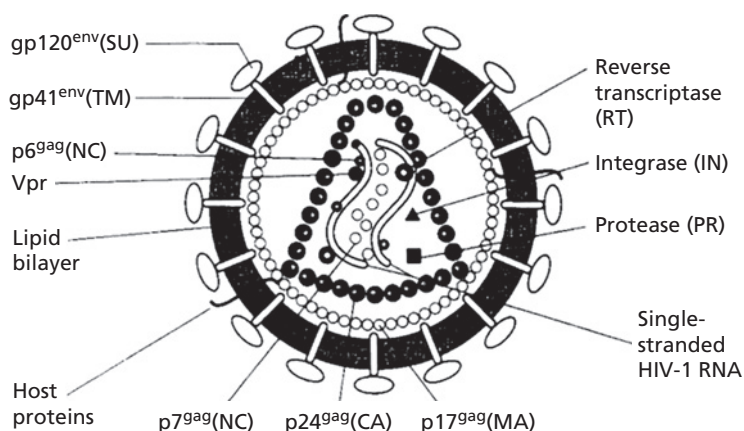


**Figure 47-2.** Schematic of the human immunodeficiency virus type 1 (HIV-1) virion. Each of the virion proteins making up the envelope (*gp120<sup>env</sup>* and *gp41<sup>env</sup>*) and inner core (*p24<sup>gag</sup>*, *p17<sup>gag</sup>*, *p7<sup>gag</sup>*, and *p6<sup>gag</sup>*) is identified. The diploid RNA genome is shown associated with reverse transcriptase (*RT*), an RNA- and DNA-dependent DNA polymerase. Integrase (*IN*) and protease (*PR*) also are present in the mature HIV-1 virion. The auxiliary protein *Vpr* is incorporated into the HIV-1 virion through interaction with the *P6gag* protein, which composes the carboxyl terminus of the *p55gag* precursor protein. CA = capsid protein; MA = matrix protein; NC = nucleocapsid protein; SU = surface protein; TM = transmembrane protein. Used with permission from Geleziunas and Greene.<sup>13</sup>

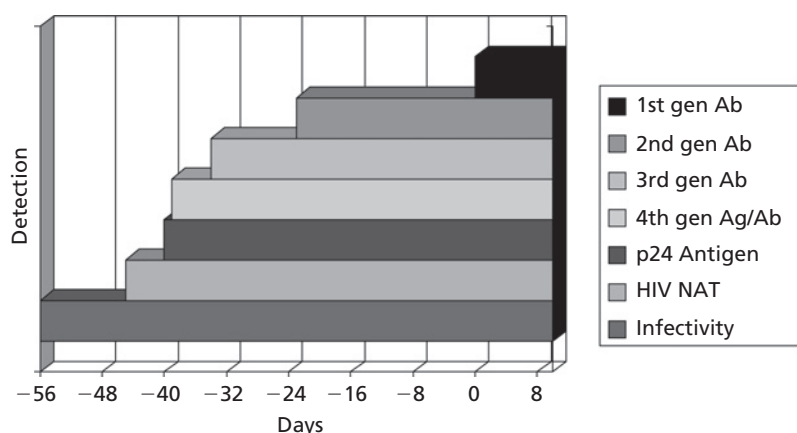


**Figure 47-3.** HIV window period detection by various HIV assays (generalized). Assuming that the first-generation HIV antibody test could detect early HIV seroconversion at Day 0, second-generation tests were able to detect seroconversion up to 23 days before, third-generation tests 34 days before, fourth-generation Ag/Ab assays 39 days before, HIV p24 antigen assays 40 days before, and HIV NAT 45 days before. This still leaves a period of around 11 days when a donor could be infective but is nonreactive in current tests.

has not increased further, although more HIV transmissions are being detected in the general population.

In the United States the incidence is reported to be 7.2 cases per 100,000 in new donors (1993 to 1996) and 2.9 cases per 100,000 in repeat donors.<sup>16</sup> In Western Europe 1.7 donations per 100,000 were HIV positive; in Eastern Europe (former USSR) this rose to 36.7 per 100,000 for the period 2000 to 2004. Ukraine had the highest prevalence with 128.4 HIV-positive donors per 100,000. The dramatic increase in the prevalence of HIV infection in Eastern European blood donors parallels the high ongoing transmission among the general populations, particularly in injecting drug users. This causes considerable concern over the safety of the blood supply in Eastern Europe.

### Donor Testing and Counseling

Shortly after the discovery of HIV as the cause of AIDS, serologic enzyme-linked immunosorbent assay (ELISA) tests for anti-HIV became commercially available during 1985 and most blood services in developed countries began screening every donation. HIV screening tests have gradually been improved over the years by replacing viral lysates with recom-

binant antigens, including HIV-2 proteins and HIV-1 group O components; using sandwich technology; and, more recently, incorporating simultaneous detection of both HIV antibody and HIV p24 antigen in the fourth-generation HIV antigen/antibody tests.<sup>17</sup> These improvements have led to a considerable shortening (almost fourfold) of the seronegative gap between potential infectivity following infection and reactivity in serologic tests (Fig 47-3). Numerous commercial HIV serologic assays exist; transfusion services select a suitable assay on the basis of sensitivity, specificity, and other criteria. Normally, the selected assay should be capable of detecting early seroconversion samples without any associated specificity problems.<sup>18</sup> External quality control samples<sup>19</sup> should be used to monitor the performance of routine screening kits and batches of test kits should also be checked to ensure sensitivity is maintained before general use.<sup>20</sup>

The advent of HIV NAT-based systems for screening blood donors (introduced in some countries in 1995) is thought to reduce the time by detection by only a few days compared with the latest fourth-generation HIV antigen/antibody test systems. Current commercial HIV NAT assays can detect 14 copies/mL



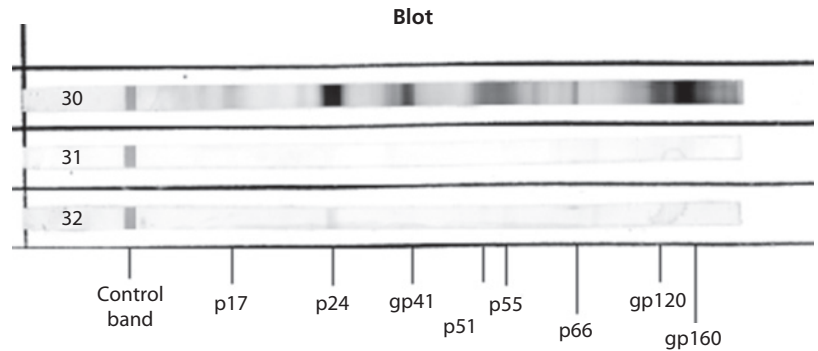


**Figure 47-4.** HIV Western blot (Genelabs HIV 2.2). Strip 30 shows a confirmed HIV-1 positive sample; strip 31 is a negative sample; strip 32 is an indeterminate (p24).

HIV-1 RNA (50% detection limit). The blood services in the United States, Canada, Germany, and France perform HIV NAT in minipools (MPs) of six to 24 donations and detect around 1 in 3 million as HIV-positive (by NAT only). Facilities in Italy observe around 1 in 450,000 and those in South Africa detect 1 in 25,000<sup>14</sup> as HIV-positive (by NAT only, serology negative). Look-back investigations on HIV MP-NAT-negative donations from donors who later were found to be confirmed HIV positive, have revealed a number of HIV transfusion transmissions,<sup>21</sup> leading to consideration of reducing the number of donations in minipools to the ultimate individual donation (ID)-NAT assay. HIV NAT should not replace serologic HIV donor screening assays, because there are occasional examples of HIV-seropositive donors with HIV viral loads below the threshold of HIV MP-NAT, being barely detectable even by HIV ID-NAT.<sup>22</sup>

Western blot testing of repeatedly reactive samples is often considered the “gold standard” for confirmation of reactivity, although reactivity in two or three alternative ELISAs of equal sensitivity is also a recognized confirmatory strategy in high-prevalence populations. Western blot displays a wide range of antibodies against various components of the virus with the identification of antibodies to p24 (core) and gp120 and gp160 (envelope) regions being essential to conclude HIV infection (Fig 47-4). However, Western blot testing has several drawbacks. It is incapable of confirming HIV infection in those who are recently infected and who may be HIV NAT-reactive or HIV p24 antigen-reactive. However, the assay invariably will produce a clear positive result on the follow-up sample from such a donor. It should be pointed out that many weak bands can appear in individuals who have never been infected. With the use of widely accepted criteria to interpret results, such “indeterminate” reactions can be considered as uninfected if no change in blot pattern is seen on follow-up samples. This position has been verified by extensive analysis of many such samples worldwide.<sup>23-25</sup>

The finding of sole HIV NAT reactivity in the absence of serologic activity requires confirmation by either an alternative HIV NAT assay or a sensitive HIV p24 antigen test (generally up to four times more sensitive than any of the combined assays).

In the absence of any confirmatory procedure, preliminary counseling of donors should be considered that includes the acquisition of another blood sample that would reveal any further development of serologic markers.

If HIV infection is confirmed, the donor should be invited to attend a clinic with a donor consultant who will advise the donor of his or her HIV status and ask for a follow-up sample for repeat testing to confirm identity. The donor would be probed regarding any potential risk situations that may have led to the infection. Use of a detuned assay on confirmed HIV-positive donors can aid in the determination whether new donors were recently infected.<sup>26,27</sup> Often donors will admit to a risk situation that they had denied when they gave their index donation. The infected donor would then be referred to the appropriate follow-up health department for clinical management.

#### Window Period and Risk of Transmission

Transfusion transmission has infected approximately 2% to 5% of those with HIV, with the vast majority of these transmissions occurring before the advent of HIV donor testing.<sup>28</sup> Around 90% of recipients of seropositive units became infected with HIV.<sup>29</sup> Since HIV screening commenced, HIV transfusion transmission has become a relatively rare event, usually associated with a blood donor in the early course of HIV seroconversion, ie, in the “window period.” This window period is defined as the time between exposure to the virus (or onset of infectiousness in the host) and the appearance of a diagnostic marker of the infection in the blood. In the late 1980s some well-documented cases of HIV-1 transmission from screening test-negative blood were reported.<sup>30,31</sup> This led to an estimate that five cases of transfusion-transmitted AIDS had occurred per year in the United States from anti-HIV-negative, early seroconversion donations. The residual risk has been estimated between 1 in 450,000 and 1 in 3 million before NAT; NAT screening has halved this risk.<sup>32</sup> The risk of transmission of HIV-2 has been estimated at less than 1 in 15 million and other subtypes are of even lesser concern. It is notable that the risk of HIV infection from blood has been reduced by almost four orders of magnitude in comparison with the transfusion AIDS epidemic of 1982 to 1984, which was responsible for around 2% to 5% of those infected

with HIV. Furthermore it has been suggested that the infectivity of a transfusion in the early 11-day window period (before HIV NAT detection) may be lower than later stages of primary infection because of the low level of virus in this eclipse phase.<sup>12</sup> It has been estimated that the doubling time of HIV is 20.5 hours in this period.<sup>33</sup> Nevertheless, despite the improvements in HIV testing, there have been reports of HIV-NAT-negative blood involved in transfusion-transmitted HIV.<sup>22,34</sup> This demonstrates that despite the employment of the most sensitive type of assay to screen blood donations, zero risk is presently still unattainable.

The residual risk of HIV transfusion-transmitted infection (TTI) has been estimated to be 0.14 per million donations for the UK blood supply during the period 1996 to 2003.<sup>15</sup> Despite improvements in testing (HIV antigen/antibody assays and HIV NAT in some centers) the residual risk has risen to 0.22 per million because the improvements in tests have been overshadowed by the gradual doubling of new infections in the general population. These new infections have been associated with heterosexual spread of genotype C and other non-B genotypes, whereas MSM spread is associated with genotype B. A similar increase in non-B HIV genotypes has been found in other European countries and, to a lesser extent, Asia and the Americas.<sup>35-38</sup> In the United States the proportion of non-B HIV-positive blood donors rose from 0.8% to 3.1% between 1993 and 2000.<sup>39</sup>

Worldwide it has been estimated that up to 13 million units are not tested for HIV, HBV, or HCV. The World Health Organization (WHO) has estimated that this would result in 160,000 TTI cases per year. More recently, the Global Database on Blood Safety stated that HIV testing is being conducted in all 152 surveyed countries, but poor quality test performance together with sporadic supply problems have contributed to HIV transmission.<sup>40</sup>

### Clinical Course

Although HIV can be inactivated by heat treatment of lyophilized products at 68°C for 72 hours, this became apparent only after a considerable number of hemophilia patients had already been infected through HIV-contaminated Factor VIII products. Indeed, around 50% of those treated with the non-heat-treated product experienced HIV seroconversion.<sup>41</sup> Initially it was hoped that those with anti-HIV were merely immunized rather than infected but studies proved that those with anti-HIV were also HIV-positive by polymerase chain reaction (PCR), indicating that they were indeed infected. This has been amply borne out by the clinical outcomes in such patients.

Because HIV can withstand the storage temperatures of blood components, it is to be expected that recipients of blood would be infected. The Transfusion Safety Study (TSS) in the United States was unique in actively tracing and enrolling recipients of known HIV-positive units. In this study 111 (89.5%) of 124 recipients of HIV-infected components experienced seroconversion to anti-HIV positivity.<sup>42-44</sup> It was noted that washed Red Blood Cell (RBC) units and RBC units stored longer than 26

days had lower transmission rates than other components. This would also suggest that leukocyte reduction might have a beneficial effect in reducing infectivity.<sup>45</sup>

Following infection with HIV, there is usually an asymptomatic period of around 2 to 3 weeks that progresses to an acute flu-like (seroconversion) illness in 40% to 90% of patients. Fever, enlarged lymph nodes, sore throat, rash, joint and muscle pains, headache, diarrhea, and vomiting are some of the signs and symptoms that can be experienced for a period of up to 2 weeks or longer. Correct diagnosis at this stage is usually unlikely unless symptoms are protracted. The infection will then become clinically latent but with active viral replication. The virus can be transmitted via blood and genital secretions during the asymptomatic and clinically latent period.

Persistent asymptomatic HIV infection lasts on average 10 years in the absence of treatment. Eventually there is a sharp decrease in the number of CD4 T lymphocytes, leading to immunosuppression and opportunistic infections that may prove fatal when not treated. When an HIV diagnosis has been made and treatment is undertaken, the many advances in HIV management can extend survival and delay the onset of AIDS. However, worldwide the majority of those infected live in developing countries where effective therapy is usually not available.

The 112 recipients enrolled in the TSS study provided valuable data as to the progression of the disease in those individuals infected through transfusion. Seven years following their transfusion, 37 (33%) had developed AIDS.<sup>46</sup> The transfusion recipients tended to have a faster rate of disease progression compared with both infected donors and infected hemophilia patients who were followed in parallel. This higher rate was mainly because the patients were older and with underlying clinical conditions.<sup>47</sup> Overall this study led to suggestions that the route of infection, size of inoculum, and other viral co-infections do not significantly alter the course of the disease. However, the importance of the viral virulence gene, *nef*, was demonstrated in an Australian cohort of a blood donor and eight transfusion recipients infected with an HIV strain lacking a functioning *nef* gene. The cohort members had a much milder illness with longer disease-free survival in the absence of therapy.<sup>48</sup>

Whether transfusion of AIDS patients is beneficial is debatable.<sup>49,50</sup> The results of the Viral Activation by Transfusion Study (VATS) showed no evidence that leukocyte reduction benefits this patient group.<sup>51</sup>

### Treatment

Antiviral treatment has evolved from monotherapy to cocktails of highly active antiretroviral therapy (HAART) that has achieved remarkable success in decreasing the mortality from AIDS and in prolonging useful active life in those with HIV infection. Three classes of antiretroviral drugs are available—nucleoside and nucleotide analogs (NRTIs); reverse transcriptase inhibitors (RTIs); and protease inhibitors (PIs). There is no cure for HIV or AIDS. It should be realized that where viral load is measured routinely, any increase may be associated with the development

of drug-resistant strains, necessitating a drug substitution in the patient's therapy.

### Human T-Cell Lymphotropic Viruses

Human T-cell lymphotropic virus (HTLV-I) was identified in 1978 when it was isolated from a Japanese patient. The virus was found to cause adult T-cell leukemia and lymphoma (ATL)<sup>52</sup> and tropical spastic paraparesis (TSP), otherwise known as HTLV-I-associated myelopathy or HAM.<sup>53</sup> The virus is now found globally but is endemic in Japan, the Caribbean, South America, and West and Central Africa, where infection rates are often above 1%. HTLV-II was originally identified in 1982 in a patient with hairy-cell leukemia. HTLV-I and HTLV-II show 65% homology. HTLV-II is found in American Indian populations and in some populations of intravenous drug users.

### Incidence and Prevalence Among Blood Donors

The overall incidence in first-time donors in the United States is around 40 per 100,000. In repeat donors a rate of approximately 1.6 per 100,000 person years was identified from 1991 to 1996. HTLV-II is found more often than HTLV-I among US first-time donors, reflecting the epidemic of HTLV-II among intravenous drug users. In the UK, routine screening of blood donations commenced in 2002 and has resulted in over 100 HTLV-positive donors being detected. However, in the UK there is a remarkable geographic variation in distribution, with southern England having an incidence as much as 10 times higher than northern regions.<sup>54,55</sup>

### Donor Testing and Counseling

Current HTLV donor screening assays are based solely on antibody detection by ELISA or chemiluminescent (ChLIA) format. The more sensitive assays have used recombinant HTLV proteins on both solid phase and conjugate in a sandwich assay format. Assays detect both HTLV-I and HTLV-II.

In the UK a lengthy pilot study was performed using anti-HTLV to test the residues of the pools of samples used for HCV NAT assays (pools of up to 95 donations).<sup>56</sup> It was estimated that this procedure was around 95% sensitive compared with individual donation testing. However, adjustment of the cut-off to enhance the test sensitivity together with reduction in the minipool size to no more than 48 donations led to an HTLV screening strategy that was comparatively inexpensive, around 98% sensitive, and with remarkable specificity (the almost ideal situation). Furthermore, the knowledge that all UK blood components were subjected to leukocyte reduction meant that this strategy carried even less residual risk of viral transmission by transfusion.

Confirmatory testing of reactive HTLV donations is more complex than confirmatory testing for HIV, partly because of the relatively poor Western blots that were commercially available. Modifications to those Western blots to include recombinant materials in addition to viral lysate and also the development of recombinant immunoblots (Fig 47-5) (similar to HCV immunoblot formats) have led to more accurate interpretations. Indeed, some of the immunoblots were capable of discriminating the

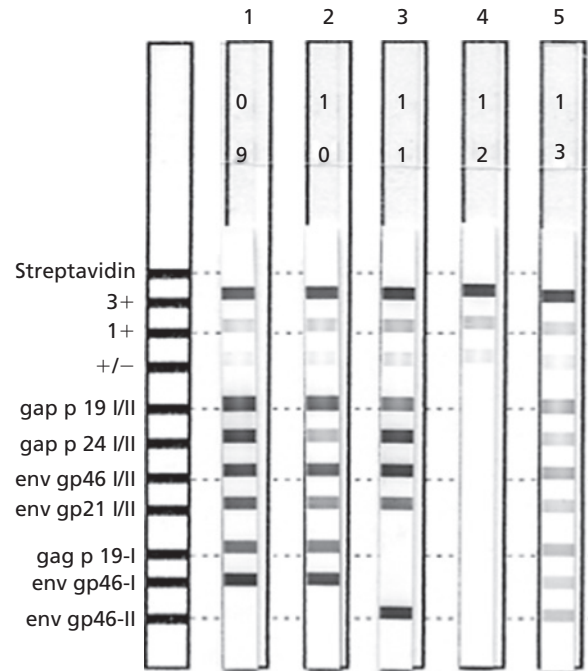


**Figure 47-5.** HTLV Immunoblot (Innolia). Strips 09 and 10 show confirmed HTLV-I specimens; strip 11 shows confirmed HTLV-II; strip 12 is a negative control; strip 13 is a positive control.

pooled donation samples (up to 95 donations) as either HTLV-I or HTLV-II positive. Nucleic acid testing as part of a confirmatory algorithm is still under development.<sup>57</sup>

Most UK HTLV-positive blood donors were shown to be of Afro-Caribbean ancestry or had been sexual partners of someone of Afro-Caribbean ethnicity. Therefore, it is important to ensure that sexual partners and other family members are also tested for HTLV in any clinical follow-up.

### Window Period and Risk of Transmission

A window period of 51 days has been calculated.<sup>58</sup> A study of US transfusion recipients estimated the risk of HTLV infection at 12 per 100,000 units before screening and 1.4 per 100,000 following the implementation of blood donor HTLV screening. Over time, this risk has changed to 0.16 per 100,000 screened donations. Generally, transfusion-transmitted HTLV has been associated with (fresher) cellular components so that plasma transfusions are thought to be of little risk. Furthermore, the use of leukocyte reduction procedures has lessened the risk considerably such that in combination with HTLV donor screening, virtual elimination of HTLV transfusion transmission has been claimed.<sup>59</sup>

### Clinical Course

Although the majority of HTLV-positive donors exhibit no clinical symptoms of disease, they should be made aware that a small percentage (2% to 4%) may develop ATL up to 40 years after infection. HAM/TSP is characterized by spinal cord degeneration and has a

Table 47-2. Human Herpesviruses

| Herpes Subfamily   | New Nomenclature | Common Name                       |
|--------------------|------------------|-----------------------------------|
| Alphaherpesvirinae | HHV-1            | Herpes simplex virus-1 (HSV-1)    |
| Alphaherpesvirinae | HHV-2            | Herpes simplex virus-2 (HSV-2)    |
| Alphaherpesvirinae | HHV-3            | Varicella zoster virus (VZV)      |
| Gammapesvirinae    | HHV-4            | Epstein-Barr virus (EBV)          |
| Betaherpesvirinae  | HHV-5            | Cytomegalovirus (CMV)             |
| Betaherpesvirinae  | HHV-6            | HHV-6                             |
| Betaherpesvirinae  | HHV-7            | HHV-7                             |
| Gammapesvirinae    | HHV-8            | Kaposi's sarcoma-associated virus |

much shorter incubation period, developing in a few (1.5% to 3%) patients infected with HTLV-I and HTLV-II. HTLV-I has also been associated with uveitis and may lead to eventual blindness.

Treatment

The management of asymptomatic HTLV infection generally should not involve drug therapy because of the low chance of clinical disease development. Chemotherapy for the treatment of ATL is often less effective than for other forms of leukemia or lymphoma. Therefore, there is a high mortality associated with this disease. HAM/TSP can be treated with systemic steroids or azathioprine immunosuppression with variable success.

Other Retroviruses

The finding of a high proportion of individuals with antibodies to the p24 core antigen on HIV Western blot is highly suggestive that these individuals may have been infected with other retroviruses. Testing sequential donations or samples from such individuals over many years has shown that their “indeterminate” banding patterns usually do not alter for a particular individual. In addition, PCR testing of such individuals has not identified any reactivity. Also, it is known that the human genome, like that of other species, harbors some ancient endogenous retroviral sequences. These observations have not resulted in any real proof of disease association.

However, there is some concern over the possibility that the use of animal-derived blood constituents could harbor such retroviruses that may re-activate following transfusion. Indeed, the latest fad of “bush meat cuisine” may also result in cross-species transmission of retroviruses.<sup>60,61</sup> Blood donor-recipient repositories in developed and developing countries will provide reassurance against such potential risks to blood safety.<sup>62</sup>

Herpesviruses

Overview

Herpesviruses are ubiquitous in nature, with practically every living creature known to be susceptible to some associated herpesvirus. Humans are known to have several herpesviruses (Table 47-2). All human herpesviruses (HHVs) have the

capability to lie dormant in tissues after an acute infection. With reference to transfusion, cytomegalovirus (CMV or HHV-5) is by far the most important herpesvirus infection. Herpes simplex (HHV-1 and HHV-2) and herpes varicella-zoster (HHV-3) have restricted tissue involvement, but CMV can manifest itself in many organs. Therefore, CMV is a major problem in immunosuppressed patients requiring transfusion therapy. Patients who should receive components that are selected or processed to minimize the risk of CMV infectivity include the following:

- Transplant recipients
- Patients with severe immunodeficiency
- Fetus (intrauterine transfusion)
- CMV-negative pregnant females
- Low birthweight premature infants and neonates

It is important to be able to distinguish between *CMV infection*, identified by means of serologic tests or viral isolation, and *CMV disease* identified by laboratory evidence of infection combined with specific symptoms associated with the virus.

Epstein-Barr virus (EBV or HHV-4) is responsible for infectious mononucleosis, is associated with Burkitt lymphoma and nasopharyngeal cancer, and can also be transfusion transmitted.

Human herpesvirus 6 (HHV-6) causes “roseola infantum” or “sixth disease”—a childhood rash. Primary infection of adults is rare.

Human herpesvirus 8 (HHV-8) has been found in Kaposi's sarcoma and other tumors such as Castleman disease and body-cavity-based lymphoma.<sup>63,64</sup> It has also been associated with polyneuropathy, organomegaly, and endocrinopathy. As with CMV and EBV, HHV-8 appears to cause a persistent infection with the viral genome, lying latent in host immune cells.

Incidence and Prevalence Among Blood Donors

Figure 47-6 shows the prevalence of CMV infection in a Scottish blood donor population with an apparent 2% CMV seroconversion rate per year. The prevalence figures for a similar population 20 years earlier showed figures that were considerably higher. Thus, it would appear that few donors had been infected in recent times (possibly because of improved hygiene and living conditions). Support for this hypothesis comes from a German study<sup>65</sup> in which an overall CMV seroconversion rate of 0.55% per year was observed.

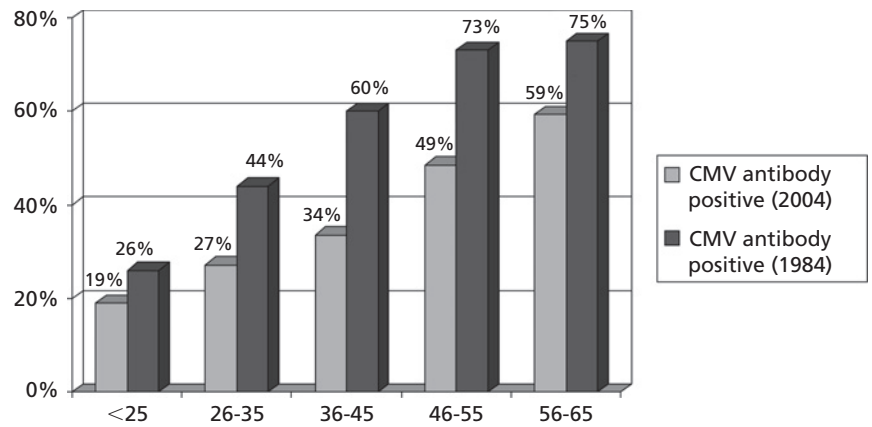


**Figure 47-6.** Comparison of cytomegalovirus antibody status of West of Scotland blood donors according to age and samples 20 years apart.

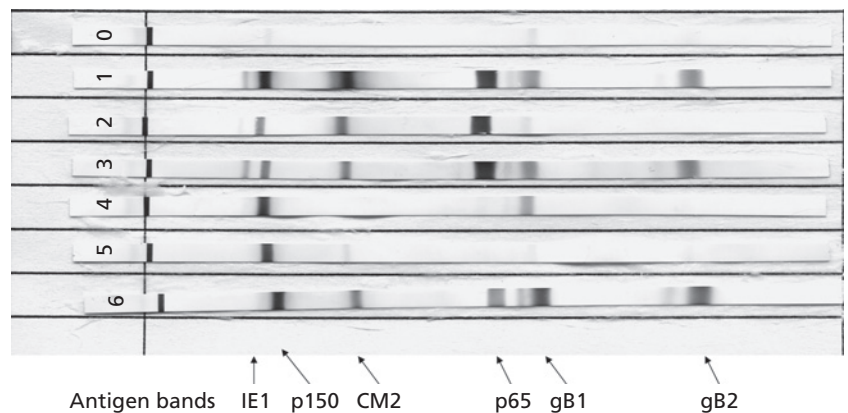


**Figure 47-7.** Western blot for cytomegalovirus antibodies showing the various antibodies that are elicited.

EBV and HHV-6 have a very high prevalence in the general population (>80%). HHV-8 has a seroprevalence in blood donors between 0 and 20%. African populations have been reported to have a prevalence of 35% to 75%, with 10% to 50% showing evidence of HHV-8 DNA. In the UK, HHV-8 has been reported more frequently in MSMs attending sexually transmitted disease clinics and this was a contributory factor in the decision to maintain permanent deferral of this population group as blood donors.<sup>66</sup>

### Donor Testing

Most transfusion testing sites use ELISA-based assays to test blood donations for the presence of anti-CMV. Unlike mandatory marker assays, anti-CMV assays are not as strictly controlled by regulatory agencies. Validation and concordance of assays is hampered by the number of antibodies that CMV can elicit.<sup>67</sup> Most transfusion testing sites tend to use “total” anti-CMV assays that can detect both IgG and IgM class antibodies.

Hemagglutination assays are inherently capable of detecting both classes of antibodies and some commercial assays have now become available that can be performed on automated blood group analyzers. In contrast, diagnostic virology laboratories use both IgG and IgM antibody tests to differentiate acute from

recrudescant CMV infections. Although it is accepted that those donors with IgM reactivity are most likely to have CMV viremia, the capacity of CMV to lie dormant in white cells means that any antibody-reactive donation has the capability of infecting the recipient with CMV. Donors with reactivity in anti-CMV screening assays usually are flagged as “CMV positive” on donor databases so that they will not be retested and their blood will not be included in the preparation of CMV-negative components. Problems associated with specificity of some ELISA tests can lead to some donors being labeled falsely as CMV positive; transfusion services are hesitant to retest these donors because of operational constraints.

Recently, a CMV Western blot assay (Fig 47-7) has become available, allowing a check to be made on samples found to show discrepant reactivity with different manufacturers’ assays. Because CMV is a common infection and not clinically significant in immunocompetent individuals, there is no need to counsel donors. No routine tests are performed for EBV, HHV-6, or HHV-8 infection. Indeed, tests for HHV-8 frequently show discrepant results.<sup>68</sup>

### Window Period and Risk of Transmission

Although CMV is transmissible by transfusion, this is now relatively uncommon in developed countries, particularly those where

all blood components are leukocyte reduced. Despite the average 3 log<sub>10</sub> reduction in leukocyte concentration achieved by leukocyte reduction,<sup>69</sup> there has been no abandonment of CMV-seronegative blood bank inventories, particularly for the patient populations at high risk of CMV disease.<sup>70</sup> It is thought that only 1% to 5% of blood units found to be CMV seropositive will actually lead to infection of CMV-seronegative recipients. The incubation period of primary CMV infection varies between 4 and 8 weeks. The residual risk of transfusion-transmitted CMV infection from anti-CMV screened blood components (not leukocyte reduced) is around 1.2% to 1.6% per donation, whereas the risk is between 2.3% and 3.0% for leukocyte-reduced blood components.<sup>71</sup> The present use of CMV screened leukocyte-reduced blood would be expected to be associated with a risk of less than 1%.

Before leukocyte reduction, EBV infection occurred between 3 and 7 weeks following transfusion in around 33% to 46% of recipients lacking EBV antibodies. Such recipients are a small minority, especially in older individuals.

The transmissibility of HHV-8 by transfusion is still debatable. Studies in the United States provided evidence of seroconversion in two of 284 cardiac surgery patients from non-leukocyte-reduced blood between 1986 and 1990.<sup>72</sup> A Ugandan study showed a 6% frequency of seroconversion with non-leukocyte-reduced blood compared with 3.5% among recipients of HHV-8 seronegative blood.<sup>73</sup>

### Clinical Course

In marrow recipients, CMV is one of the most significant infections, responsible for 15% of mortality before the advent of good antiviral drugs. CMV matching of kidney recipients has a beneficial effect on clinical outcome more so than matching patients for HLA Class I status. The use of seropositive blood for seropositive patients can lead to reinfection of some CMV seropositive recipients; therefore, CMV-seronegative components are preferred (but not always available) for all immunocompromised patients. The leukocyte reduction of blood components has lessened the demand on CMV-seronegative components.

Transfusion-transmitted EBV infections usually are asymptomatic, but some appear as an infectious mononucleosis with mild fever, anorexia, and malaise. Most HHV-8 infection is benign with a low frequency of disease development.

### Treatment

Antiviral drugs that are effective against CMV (and also HHV-6) include ganciclovir, foscarnet, cidofovir, and valganciclovir. Drugs can be prescribed either for prophylaxis, suppression, or treatment.

## Other Viruses

Several viruses have had a considerable impact on blood services in certain parts of the world. These “other” viruses include parvovirus B19 (human erythrovirus), West Nile virus, chikungunya, and pandemic influenza.

### Parvovirus B19

Parvovirus B19 was so named because it was the 19th sample on a hepatitis B counter electrophoresis assessment panel that reacted; subsequently it was shown to contain parvovirus. Parvovirus is a single-stranded nonenveloped DNA virus of 5500 nucleotides that code for two capsid proteins (VP1 and VP2) and other proteins.<sup>74</sup> Parvovirus is the agent that causes “fifth disease” or “slap-cheeked syndrome” in young children. However, parvovirus can also cause serious, even life-threatening, disease in certain immunosuppressed individuals and women in the second trimester of pregnancy, where hydrops fetalis may result. Individuals lacking the P blood group globoside are naturally immune to parvovirus because the virus requires the globoside for binding to the cell. Various cell types contain this surface antigen and are susceptible to infection. The clinical manifestations of parvovirus are, therefore, widespread, involving fetal myocardium, placenta, liver, kidney, thyroid, megalokaryocytes, and erythroid progenitor cells.

Compared to some blood derivatives, blood components are only rarely associated with parvovirus B19 transmission. This may be because of a lack of recognition of an association of parvovirus infection with transfusion or, more likely, asymptomatic infection in the recipient. Nevertheless, recent studies suggest between 0.5% and 0.9% of individual blood donations are contaminated with parvovirus B19 by evidence of B19 DNA. It could be argued that other components containing antibodies may prevent infection in some recipients, but ongoing studies with an archive repository in the United States may reveal the true transmission rate. Various parvovirus serologic assays are now commercially available.<sup>75</sup> The Netherlands have reacted to the risk of parvovirus infection in selected patient groups by providing a “parvovirus-safe” panel of donors deemed to be safe from transmitting parvovirus by evidence of parvovirus antibodies for a period of at least 6 months. This panel was created by large-scale ELISA screening of the donor panel for parvovirus antibodies (against VP2) over a period of over a year. The demand for “parvovirus-safe” blood components has been limited. Recent studies<sup>76,77</sup> raise doubt over the strategy, as they describe low-level parvovirus B19 carriage in individuals with cocirculating antibodies.

In the past, parvovirus B19 transmissions have occurred through plasma-derived Factor VIII or IX blood derivatives. Although parvoviruses are generally stable and resist most physicochemical inactivation procedures, parvovirus B19 is more readily inactivated than the other parvoviruses using either dry or wet heat, extreme pH, ultraviolet C irradiation, photochemical reaction, or even nanofiltration procedures in blood derivative manufacture. Despite the incorporation of some of these inactivation procedures and the use of recombinant-derived clotting factor concentrates, reports of potential parvovirus B19 transmission with high-purity clotting factor concentrates, immunoglobulin preparations, and solvent/detergent-treated plasma still occurred. This led plasma fractionators worldwide to screen their source plasma for parvovirus DNA and use only pools with less than 10,000 copies/mL. This level of reactivity was ascertained by considering the level

of parvovirus antibodies that were also contained within these pools and its ability to neutralize small concentrations of the virus. A pilot study on parvovirus NAT testing was able to demonstrate some exceedingly high viral concentrations in maxipools of donations. The level of parvovirus mirrored the level of infection in the general population where epidemics appear every 3 to 4 years, usually in late spring.<sup>78</sup> The prevalence of parvovirus is age related, with most infections occurring in young children, who often then infect their (non-immune) siblings, parents, or grandparents. By the age of 15 years, 60% of the population will possess antibodies to parvovirus.<sup>79</sup> Most parvovirus assays detect three genotypes, Parvovirus 1, 2, and 3. Two new parvoviruses, Parvovirus 4 and Parvovirus 5, have been described with no known pathogenicity.<sup>80</sup>

### West Nile Virus

West Nile virus, a flavivirus, was first recognized in 1937 and now has a wide distribution in Africa, Europe, Asia, Australia, and, in the past decade, in Northern America. WNV mainly infects birds and is normally transmitted by mosquito. However, horses and also humans can be bitten by infected mosquitoes and subsequently can develop West Nile fever.<sup>81</sup>

In the United States, WNV was first identified in 1999 and over the next few years spread from the eastern seaboard to the Pacific states. Most human cases occur between July and November with a peak period in August and September, but viremic blood donors have been found throughout the year. Cases in the United States peaked in 2003 with 9862 cases and 264 deaths, but WNV is likely to continue to cause around 2000 cases and 100 deaths per year.

The incubation period is between 3 and 15 days in humans with over 80% of infections being asymptomatic or with a mild flu-like illness. In rare cases (fewer than 1% of those infected), a more severe disease results and fatal encephalitis can occur. Asking donors about predonation headache and fever was found to have no effect on blood safety.<sup>82</sup> The risk of transfusion transmission is associated with a period of viremia occurring around 3 days after infection and lasting around a week.<sup>32</sup> Since July 2003, the US and Canadian blood supply has been screened using WNV NAT assays that were developed quickly by two major diagnostic companies, Roche and Chiron.<sup>83</sup> Both assays can be used on minipools of six or 16 donations. However, following reported WNV transmissions from MP-NAT-negative donations, an algorithm was devised to switch to ID-NAT when there was demonstrable WNV activity in a region.<sup>84</sup> This strategy seems sensible, particularly because 294 (22%) of 1332 viremic blood donors tested during the period 2003 to 2005 were detected solely by ID-NAT.<sup>85</sup>

Following an outbreak in horses in southern France, a survey of 2157 blood donors in that region showed that 0.8% had evidence of antibodies to WNV.<sup>86</sup> Various outbreaks in Europe have not equalled the WNV epidemic in the United States. Perhaps the American WNV is more pathogenic, or alternatively, perhaps Europeans are less susceptible. European blood services have found that deferring donors from North America for a period of 28 days offers sufficient protection to their blood supply. Fresh frozen

plasma imported from the United States is currently pathogen inactivated (eg, with methylene blue) to combat any WNV risk.

### Chikungunya

Chikungunya virus (CHIKV) is an alphavirus of the *Togaviridae* family with a single-stranded RNA genome. The virus is transmitted by mosquitoes (*Aedes albopictus*) to humans, where after 1 to 10 days symptoms of chikungunya fever develop. Derived from the Swahili name for “that which bends up,” Chikungunya disease symptoms include arthralgia, myalgia, rash, headache, and fever. Three main genotypes exist associated with either West Africa, Central and East Africa, or Asia. More recently, there has been an epidemic associated with the Central and East African genotype that was first reported in 2005 in French Indian Ocean islands, but subsequently spread to cover most Indian Ocean islands and the Indian subcontinent. It has been estimated that at the height of the epidemic over 250,000 (35% of the population) had been infected on Reunion Island, with 250 deaths. French blood service authorities originally managed the problem by importing blood to the French territories as a short-term measure, with the implementation of chikungunya NAT testing and pathogen inactivation as longer-term measures.<sup>14</sup> Blood services outside the affected areas were able to defer (for a period of 28 days) those donors who had visited areas where chikungunya was known to be epidemic.

During 2007, European blood services realized that foreign travel can aid the spread of chikungunya not only as isolated cases in returning visitors but also as an outbreak (eg, chikungunya fever in an Italian province). The latter event occurred after a visit to the region from a foreigner incubating the disease, together with the spread of the disease by the *Aedes albopictus* mosquito within that region. This mosquito species is also capable of transmitting dengue virus and is found elsewhere in Italy, France, and Albania. This highlights the need for continued vigilance of emerging infections elsewhere in the world, particularly because world travel can quickly transfer disease from one area to another. In addition, the effects of global warming allow insect vectors to survive in what once were less temperate regions.

### Pandemic Influenza

Pandemic influenza has been a continuing threat arising shortly after the severe acute respiratory syndrome (SARS) outbreak. The lethal influenza viruses that have infected humans have been adequately managed so far by existing public health measures.<sup>87</sup> This has been helped by the relative inability of “bird flu” viruses to infect humans. However, concern exists that eventually a mutation or a mixed infection will occur that will allow one of these lethal viruses to spread among humans. Because there have been a number of bird flu cases in humans involving the influenza subtype H5N1, it is felt that any new emerging influenza threat is likely to be closely related. Vaccines have been prepared, but as with normal human influenza vaccines, they have good efficacy in immunocompetent individuals but are of limited use among those who are at greatest risk from such a pandemic (ie, the old and the very young). It is believed that transfusion-transmitted influenza is relatively rare,<sup>88</sup> but theoretically



possible. Thus, the deferral of donors from areas at the height of an epidemic may be the safest option, along with the backfilling of blood stocks from areas with few or no cases. At the height of any epidemic it is likely that the use of blood will be severely curtailed—partly because of cancellation of elective surgery, but also because of a lack of health-care personnel and, indeed, blood donors.

### Lymphocytic Choriomeningitis Virus

Lymphocytic choriomeningitis virus (LCMV) was first described in 1931 as one of the main causes of meningitis. Eight solid-organ transplant recipients have been infected with LCMV from two organ donors,<sup>89</sup> raising the concern that LCMV may also be transfusion-transmitted despite a lack of reported cases in the literature. A French study<sup>90</sup> ascertained a low (0.33%) prevalence in the blood donor population, suggesting that the risk of transmission is low. However, the researchers did suggest that history of recent rodent contact should lead to a temporary deferral of potential donors.

### Simian Foamy Virus

Strategies for limiting the spread of simian foamy virus are similar to those used to manage LCMV. Donors who have occupational exposure to primates have been deferred in Canada as a measure to prevent potential transmission.

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## Emerging Nonpathogenic Viruses

The use of new powerful molecular genetic techniques to identify previously undescribed viruses has led to a plethora of “new” viruses that have yet to be shown to have any human pathogenicity.<sup>91</sup> Two such viruses have instigated much research and literature in the past 20 years—GB virus-C (GBV-C, once known as hepatitis G virus) and TT virus (TTV).

### GBV-C

GBV-C is an enveloped RNA virus and a member of the *Flaviviridae* family (which also includes HCV). The viral genome is structured similarly to HCV but has only 25% homology with it. Several genotypes exist, mainly confined to various continents.

Transfusion transmission of GBV-C has been demonstrated. The prevalence of GBV-C RNA in blood donors ranges from 1% to 5% dependent on geographic location. Antibody as measured by anti-E2 is usually found three to four times more frequently than is viral RNA.<sup>64</sup> GBV-C, and independently “hepatitis G virus (HGV),” was discovered in patients with sporadic “non-A, non-B” hepatitis, inferring that it was a causal agent of hepatitis. However, subsequent investigations have concluded that GBV-C was not the cause of viral hepatitis, nor had any hepatotropic or other pathologic role. It was merely a “passenger” virus, isolated by chance. Blood screening is therefore not necessary.

### TT Virus

As with GBV-C, TT virus was discovered during the search for the elusive cause of the minor (non-C) component of “non-A through

E” posttransfusion hepatitis. The virus was discovered in a post-transfusion hepatitis patient with the initials TT. TTV is a small (18-50 nm) DNA nonenveloped virus. TTV can be transmitted by transfusion and has a TTV RNA prevalence of between 5% and 80%; there is again a marked geographic variation in infection rates with higher prevalence in Africa (up to 80%).<sup>64</sup> It is thought that 50% of African children will have been infected by TTV by 1 year of age. More sensitive NAT assays detect higher rates of viral infection. The relevance of TTV in transfusion is limited because of the lack of any clinical symptoms. However, the fact that some current fractionated plasma product inactivation procedures are unable to prevent infection of recipients is of some concern.

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## Conclusion

The pathogenicity of any virus is extremely important when considering the value of introducing specific blood safety interventions. Another issue is the difficulties of testing and excluding infected donations when the prevalence of an agent in the donor population is high. Nevertheless, there are pragmatic actions that can be taken to reduce the likelihood of “at-risk” recipients being given infected blood. The blood supply will never be deemed to have “zero risk,” but there is an expectation by the public for blood services to provide blood that is as safe as possible. This will include steps such as excluding “high-risk” donors temporarily or permanently, using serologic or NAT-based assays to identify donors infected with pathogenic blood-borne agents, or using pathogen inactivation/reduction techniques on particular blood components to ensure that there are considerable reductions in microbial infectivity (see Chapter 51). The latter intervention is likely to be a potential basis for blood safety strategies to avert the emerging diseases of the future. Effective microbial inactivation of the full inventory of blood components would be one prerequisite of such a strategy. The case to discontinue the use of any of the present arsenal of tests/procedures will be unlikely to succeed unless the inactivation procedure is extremely reliable and robust. Commercial companies promoting pathogen inactivation systems suggest that the cost savings realized with discontinuing CMV and bacterial testing will more than adequately compensate for the costs of introducing their systems, claimed to be effective against nearly all transfusion-transmissible agents.

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## Disclaimer

The authors have disclosed no conflicts of interest.

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The risk that a blood recipient in the United States will receive a component contaminated with parasites is, in absolute terms, small but not negligible. Estimates covering nearly 20 years are that roughly one per million transfusions might transmit parasitic infection.<sup>1,2</sup> Given the large increase in international travel by US residents, the movement of large numbers of military personnel, and growing immigration, the opportunity for human trafficking of parasites that are endemic in tropical countries has never been greater. With the further development of molecular tools and improved diagnostics, these risks are being clarified. For some parasites, including *Babesia* and *Leishmania* species, the risk has probably been underappreciated given that transfusion transmission can easily go undetected, especially when the recipients are immunocompetent patients. The residual risk for some protozoan parasites appears to be on par with, or even greater than, transfusion-transmitted viral infections for which nucleic acid screening is performed. This chapter discusses the parasitic agents most likely to be found in blood donors, the risks from each, and the strategies for eliminating or diminishing these risks.

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### Chagas' Disease

Concern for transfusion transmission of *Trypanosoma cruzi* infection has increased in recent years, concurrent with the growing Hispanic population in the United States. Endemic in Central and South America and in Mexico, this protozoan hemoflagellate is transmitted in nature by triatomines, or reduviid bugs (also called "kissing bugs" for their tendency to bite on the face). Trypanosomes are not injected through the bite of the insect vector; rather, the insect vector deposits the infective metacyclic forms of the trypanosomes in its excreta during or soon after the blood meal. The sleeping host, in scratching the wound, can rub the

parasite into the wound or transfer it to mucous membranes in the eye or mouth. Triatomids competent for transmitting *T. cruzi* are widespread, including in the United States, where rare autochthonous transmission has been documented.<sup>3</sup> The agent can also be transmitted readily by blood transfusion and solid-organ transplantation, and by congenital transmission.<sup>4</sup> Rare outbreaks from contaminated food or drinks have also been reported.<sup>5</sup>

The typical clinical course following infection is an acute phase lasting 4 to 6 weeks, characterized by mild symptoms including fever, malaise, and edema of the face, as well as lymphadenopathy and hepatosplenomegaly.<sup>6</sup> The fatality rate in the acute phase is usually less than 5%, and many cases go unrecognized. During this period, parasites are readily detectable in peripheral blood.<sup>4</sup> In the ensuing indeterminate phase, however, carriers remain asymptomatic, and parasitemia tends to be low grade and intermittent.<sup>7</sup> Up to decades later, 15% to 30% of carriers will develop serious sequelae involving the heart and gastrointestinal tract (mega-colon and mega-esophagus). Chagas' disease kills an estimated 13,000 people annually, mostly from cardiac complications.<sup>4,8</sup> Acute-stage treatment with benznidazole or nifurtimox is effective, but treatment during the chronic phase has only recently been shown to be effective. A 60-day course of benznidazole treatment eliminated infection in nearly two-thirds of chronically infected children,<sup>9</sup> while a study in Argentina suggested that chronic-phase treatment with benznidazole might slow or prevent progression to cardiac disease.<sup>10</sup>

Despite considerable success in reducing insect transmission in the last two decades, Chagas' disease remains a major public health problem in many countries, with an estimated 9 million infected and 25 million at risk for infection.<sup>11</sup> The Southern Cone Initiative, inaugurated in six South American countries in 1991, sought to eliminate domestic infection by *Triatoma infestans* (the primary vector in several countries) and to interrupt transfusion transmission through universal donor screening.<sup>7</sup> Estimated annual incidence of cases has declined by 90%, and Chile, Brazil, and Uruguay are all certified as having interrupted vector transmission.<sup>12</sup> The success of this initiative inspired similar efforts in four Andean countries,<sup>13</sup> Central America,<sup>14</sup> and, the Amazon

region.<sup>15</sup> Different species of vectors and different ecologic conditions apply across these regions, so no universal strategy can be applied uniformly.

The adoption of universal blood screening in Latin American countries has dramatically altered the risk for transfusion transmission in countries where *T. cruzi* is endemic. Whereas in the early to mid-1990s only four of 17 Latin American countries in which data were compiled had achieved universal donor screening, 10 of 17 were at or above 99% coverage by 2002.<sup>16</sup> During this period, estimates of cases of transfusion-transmitted *T. cruzi* in those countries dropped by nearly 75%, from 1996 to 536.<sup>16</sup> Mexico alone accounts for two-thirds of recent estimated cases because of its large number of donors and relatively low screening coverage (27%), despite a moderately low seroprevalence in donors. A recent evaluation in Mexico demonstrated four cases of transfusion transmission and estimated that up to 1800 such cases occur in Mexico annually, on the basis of state-specific prevalence rates and donation data.<sup>17</sup>

Clearly, the risk in the United States and other countries receiving Latin American immigrants depends on the immigrants' risk for acquisition of infection extending decades into the past. US census figures estimate that 41 million Hispanics live in the United States, of whom two-thirds are of Mexican origin,<sup>18</sup> 2.9 million are of Central American origin, and 2.2 million are of South American origin. Of these, an estimated 40%, 68%, and 70%, respectively, were foreign-born. Among the foreign-born, 40% or more arrived in the United States before 1990,<sup>18</sup> and the subsequent intensification of control programs in *T. cruzi*-endemic countries. This suggests that a very large number of Hispanic immigrants might have been exposed long before entering the United States. Recent estimates project that between 70,000 and 100,000 Hispanic immigrants in the United States may be infected with *T. cruzi*.<sup>19</sup> Canada and Australia are estimated to have more than 1000 *T. cruzi*-infected immigrants each, and Spain is estimated to have more than 10,000 undetected infections.<sup>20</sup> Within the United States, 50% of Hispanic residents live in California and Texas, and a total greater than 25 million live in those two states plus Florida and New York. In all, about 80% of Hispanics in the United States live in nine states, but risk for transfusion transmission of *T. cruzi* can be found countrywide.<sup>18</sup>

There have been seven reported cases of *T. cruzi* transfusion transmission in the United States and Canada,<sup>21</sup> and five cases of transmission associated with solid-organ transplantation.<sup>22,23</sup> The actual number of transfusion cases is undoubtedly higher, because all seven cases have been detected in immunocompromised patients.<sup>21</sup> Although platelet products have been implicated in six of these seven events, the study in Mexico<sup>17</sup> revealed that two of four recipients who became *T. cruzi*-positive following transfusion had received whole blood from radioimmune precipitation assay (RIPA)-positive donors. An in-vitro study confirmed survival of *T. cruzi* up to 3 weeks in Whole Blood and Red Blood Cell (RBC) units,<sup>24</sup> underlining that the risk is not limited to platelet products. The largest look-back study performed in the United States found zero positive

recipients of 18 tested; only three of the components were platelets.<sup>25</sup> It remains unclear whether the apparently higher risk from platelets results from greater parasite survival and infectivity in this component or from the greater likelihood that recipients of platelets might be immunocompromised. Overall, data are lacking on infectivity estimates for different components, but it is estimated that roughly 20% of contaminated Whole Blood units will result in transfusion transmission.<sup>7,26</sup> Preliminary look-back efforts following introduction of a screening test in the United States suggest infectivity as low as 10% for RBC units.<sup>27</sup>

There is no definitive estimate of the nationwide seroprevalence of *T. cruzi* antibodies in the United States, but a study of donors in Miami and Los Angeles indicated roughly 1 per 10<sup>4</sup> donations were from a seropositive donor, and in Los Angeles this risk appeared to be increasing over time.<sup>25</sup> More recently, nearly 150,000 donors were screened as part of the licensure of a new commercial enzyme-linked immunosorbent assay (ELISA) for *T. cruzi* screening of blood donors. Conducted in three sites across California and Arizona, the repeat-reactive rates ranged from roughly 1:2000 in Los Angeles to 1:6000 in Tucson with half of all repeat-reactive donations testing positive by RIPA, a confirmatory assay, which at that time was not licensed by the Food and Drug Administration (FDA).<sup>28</sup> Following FDA licensure of the screening test in December 2006,<sup>29</sup> blood collection centers representing two-thirds of the US collections reported at least 582 RIPA-confirmed donors through August 8, 2008. Post-licensure results from two large blood centers covering a broad geographic area suggest an aggregate donor prevalence around 1:21,100.<sup>31</sup> Figure 48-1 reflects that although the states of California and Florida account for the great majority of RIPA-positive donors, the risk for transfusion transmission of *T. cruzi* is evident countrywide. Follow-up studies will help clarify the relationship between serologic status and parasitemia in donors, and the infectivity of components. Regulatory guidance is pending regarding donor screening for Chagas' disease, but the FDA has in the past publicly declared its support for universal donor testing given the availability of appropriate screening tests.<sup>32</sup> Meanwhile, AABB has issued recommendations for product management, recipient testing, and donor referral for medical evaluation for those blood centers choosing to implement Chagas' screening in the absence of FDA requirements.<sup>32</sup>

Aside from testing, options for risk reduction include donor exclusion, pathogen removal, and pathogen inactivation. Canada has for years addressed risk by excluding donors on the basis of residence and travel history in areas where the disease is endemic, but intends to implement the newly licensed ELISA.<sup>33</sup> In any case, deferral of donors on this basis is likely to be feasible only where such donors constitute a small portion of the donor pool. Leukocyte reduction filters have been shown to lower the concentration of trypanosomes in blood,<sup>34</sup> but at least one case of transfusion transmission in platelets was from a leukocyte-reduced and irradiated product.<sup>21</sup> A variety of photochemical treatment methods in platelets,<sup>35</sup> plasma,<sup>36</sup> and red cell components<sup>37</sup> all show promise in reducing the parasite load by several logs.

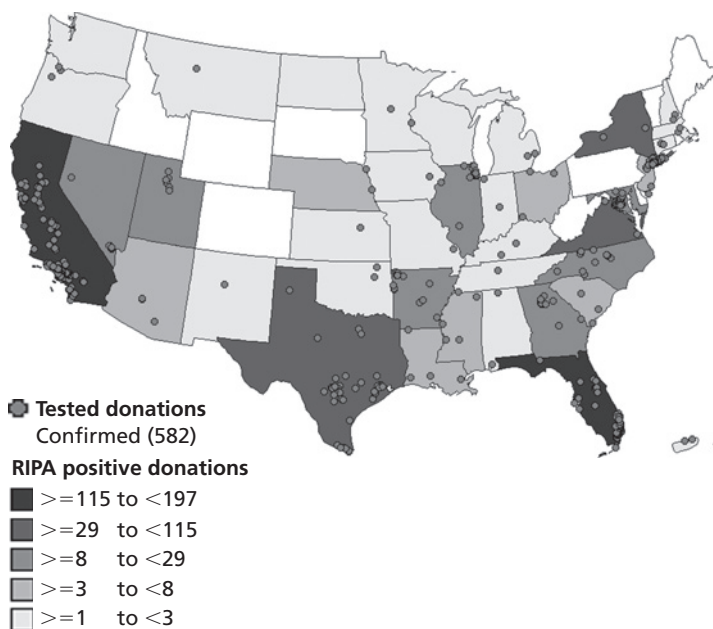


**Figure 48-1.** Donors with confirmed *T. cruzi* infection are concentrated in states with high numbers of Hispanic residents, but the risk for transfusion transmission exists countrywide. Data through August 8, 2008.<sup>30</sup>

## Malaria

Human malaria is caused by one of four species of protozoan parasites: *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. Malaria is a significant cause of global morbidity and mortality, responsible for an estimated 350 to 500 million clinical cases annually and over one million deaths; the disease is especially severe among young children in sub-Saharan Africa.<sup>38</sup> Natural infection with malaria typically occurs through the bite of an infective female anopheline mosquito. Less frequently, parasites might be transmitted through blood transfusion, organ transplantation, parenteral exposure, or mother-to-child transmission.<sup>39,40</sup> The United States recorded an average of three cases of transfusion-associated malaria per year between 1963 and 1989.<sup>41</sup> An average of about one case per year—less than 0.1 per million donations—was the rate of occurrence from 1990 to 2005.<sup>42</sup> This compares favorably to the historical estimate of zero to two cases per million donations in nonendemic areas, as contrasted with an incidence of 50 cases per million donations in areas where malaria is endemic.<sup>43</sup>

In malaria-endemic settings, where frequent boosting by infective mosquito bites help induce and maintain protective immune responses, severe infection is rare for those who survive beyond early childhood.<sup>44</sup> In a nonimmune population, such as that of the United States, clinical malaria presents as a febrile illness with paroxysms, possibly at regular intervals, with accompanying flu-like symptoms. Complications can include severe anemia, hepatic involvement, cerebral alterations, renal failure, and shock.<sup>40</sup> The case-fatality rate for malaria is generally

low—less than 1% in developed countries,<sup>45,46</sup> but can surpass 10% in recipients of blood components.<sup>41</sup>

Once endemic throughout large parts of the country, malaria in the United States is now almost exclusively caused by imported infections.<sup>47</sup> In a typical year, 1200 to 1500 malaria infections are diagnosed in the United States, more than 95% of which are found in US civilians who have visited malaria-endemic areas and in foreign civilians from malaria-endemic countries. A comprehensive review of transfusion-transmitted malaria in the United States indicates that many of these infections were preventable, with >60% having occurred because of deviations from the prevailing deferral guidelines.<sup>41</sup> Of the cases in which donor exclusion guidelines were followed, *P. malariae* is 15 times more prevalent than its share of imported malaria overall in US travelers: 65% to 4%, respectively. This fact reflects the ability of *P. malariae* to remain at subpatent levels for lengthy periods, even up to 40 years.<sup>48</sup> Indeed, because all malaria parasites remain viable in blood stored at 4°C for at least 1 week, there exists risk from all four species.<sup>39</sup> *P. falciparum* malaria has been transmitted in blood stored for 19 days, as well as in units of platelets.<sup>49</sup> Interval to onset in transfusion cases is likely to vary by species, with *P. falciparum* being the quickest to develop at about 10 days, *P. vivax* taking 16 days, and *P. malariae* taking 40 days or more.<sup>39</sup> Where a donor has been implicated, recent history in the United States implicates presumably semi-immune visitors from malaria-endemic areas. Over a 20-year period, only one US civilian without previous residence in malaria-endemic countries has been implicated in transfusion transmission, in contrast to 28 former residents of malaria-endemic countries.<sup>41</sup>

In the absence of an FDA-approved blood screening assay, prevention of transfusion-transmitted malaria in the United States has long relied on exclusion of presenting donors with elevated risk for malaria infection. The different categories, which follow guidelines of FDA<sup>50</sup> and AABB,<sup>51</sup> are as follows:

1. Donors who are permanent residents of countries where malaria is not endemic who travel to an area considered malaria-endemic by the Centers for Disease Control and Prevention (CDC) are not accepted as donors of whole blood and blood components until 1 year following departure from the malaria-endemic area, assuming they have remained free of malaria symptoms.
2. Prospective donors who have had malaria should be deferred for 3 years after symptoms have abated.
3. Immigrants, refugees, citizens, or residents of countries where malaria is endemic should not be accepted as donors of whole blood and blood components before 3 years after departure, assuming they have remained free of malaria symptoms.

These guidelines do not apply to plasma, or plasma components or derivatives lacking intact red cells. In 2000, the FDA released draft guidance<sup>52</sup> that contained the following modifications to the 1994 policy:

1. Clarified the term “resident” of a malaria-endemic country as applying to those who had resided in a malarious area for 5 years or longer.
2. Clarified that prospective donors who have had malaria must have received appropriate treatment before remaining asymptomatic for 3 years.
3. Stipulated that donors who might possess partial acquired immunity to malaria should not be accepted as donors of whole blood or blood components for a period of 3 years following their last visit to a malarious region.
4. Called for specific donor travel questions, including capture questions with follow-up of travel history.

This 2000 draft guidance remains pending, and may be superseded by different recommendations discussed at a March 15, 2001 Blood Products Advisory Committee meeting.<sup>53</sup>

Advances in diagnostics have created opportunities for non-malarious countries to implement strategies that lessen the impact of malaria deferrals while minimizing risk of transfusion transmission. In Europe, regulations<sup>54</sup> published by the Council of Europe in 2006—and updated since then—endorse the use of validated immunologic tests to shorten the deferral period of donors with potential malaria risk who test negative. Those donors who have lived for 6 months or more in a malaria-endemic area are acceptable as blood donors if they test antibody-negative at least 4 months after their last visit to such an area, as opposed to being permanently deferred. Likewise, donors who report a travel history <6 months duration to malaria-endemic area may be accepted as blood donors if they test antibody-negative at least 4 months after their last visit to such an area, in contrast to a 1-year (no malaria symptoms reported) or 3-year deferral (malaria-like symptoms within 6 months of return from malarious area).<sup>54</sup>

Since 2001, England has used an enzyme immunoassay (EIA) based on three recombinant *P. falciparum* antigens and one *P. vivax* antigen (all to erythrocytic merozoite stage) to shorten the deferral period for at-risk donors to 6 months for those testing negative.<sup>55</sup> Australia also seeks to reinstate deferred donors on the basis of this same test,<sup>56</sup> and several other countries have malaria donor screening tests under evaluation.<sup>43</sup> Although not all four *Plasmodia* species are screened for in the assay adopted by England, it accounts for the two species causing most cases of transfusion transmission and does appear to have some cross-reactivity with other species.<sup>57</sup> Immunofluorescent antibody tests for malaria antibody detection tend to be sensitive, but have the limitations of being time-consuming and subjective. Direct methods for detection of malaria infection are also available. Most widely used for malaria diagnosis worldwide is Giemsa- or Wright-stained blood films, although their use is not practical for mass screening in areas where malaria is not endemic, where labor costs are prohibitive, and the expertise is lacking. Assays detecting circulating parasite antigens such as histidine-rich protein 2 and lactate dehydrogenase offer sensitivity as low as 100 to 1000 parasites per microliter,<sup>43</sup> still insufficient to prevent transfusion transmission. A variety of nucleic acid amplification test (NAT) techniques allow for detection of one or more malaria species. Conventional<sup>58</sup> and semi-nested polymerase chain reaction (PCR)<sup>59</sup> screening of blood bank samples have detection thresholds on the order of  $10^{-3}$  parasites/ $\mu$ L. Evaluation of the costs of screening blood donors by PCR supports a strategy targeting only those donors with identified risk factors for malaria by questionnaire, not one based on universal screening.<sup>60</sup> Newer NAT techniques such as loop-mediated isothermal amplification assay offer sensitivity and specificity equivalent to real-time PCR, but with shorter turnaround time and lower costs.<sup>61</sup> With an infectious dose theoretically as low as one to 10 parasites per unit of blood, however, even the most sensitive NAT detection methods cannot reduce the risk to zero.

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## Babesiosis

Awareness of the risk for transfusion-transmitted babesiosis is growing, though historically it has been underappreciated. Transmitted by ticks, over 100 species of these intra-erythrocytic protozoan parasites infect a large number of vertebrate species worldwide.<sup>62</sup> In the United States, most reported cases of human babesiosis are caused by *Babesia microti*, especially in the Northeast and Upper Midwest; in Europe, *B. divergens* is considered responsible for most human illness from babesial infection. Heightened scrutiny, together with growing phylogenetic analyses, is documenting the human risk in new areas, as well as demonstrating a greater diversity of infecting species than heretofore appreciated.

Clinical manifestation of *Babesia* infection covers a broad spectrum, ranging from chronic, silent infection to fulminant, malaria-like illness. Most *B. microti* infections appear to be sub-clinical, but mild flu-like symptoms including fever, headache,

and myalgias can occur 1 to 6 weeks following a tick bite.<sup>62</sup> Risk for severe disease is higher in the elderly, the immunocompromised, and the asplenic. Complications can include thrombocytopenia; hemolytic anemia; and renal, heart, or respiratory failure.<sup>62</sup> The case fatality rate can range from 5% to 10% for *B. microti*<sup>63</sup> to 29% for *B. divergens*.<sup>64</sup> All reported cases of *B. divergens* have occurred in asplenic hosts,<sup>64</sup> which almost certainly contributes to this disparity.

Since the first case of human babesiosis was recorded in the United States in 1968,<sup>65</sup> over 1500 cases have been reported in the country. The primary agent *B. microti* is transmitted by the black-legged deer tick (*Ixodes scapularis*, formerly known as *I. dammini*), the vector of Lyme disease. Most US cases are reported in the Northeast and the Upper Midwest, with the former region extending as far south as New Jersey. Sporadic cases of human babesiosis caused by other *Babesia* species have been reported from Washington state and northern California, Missouri, and Kentucky since the early 1990s.<sup>66-68</sup> In Europe, about 30 human cases of babesiosis have been reported since the first report in 1957, a fatal case in an asplenic Yugoslav farmer.<sup>69</sup> Most European cases are attributed to *B. divergens*, also a bovine pathogen, with cases reported from several countries in Western Europe, the former Yugoslavia, and the former USSR.<sup>64</sup> Risk for human infection correlates geographically with the presence of infected cattle populations and areas infested by the tick vector, *Ixodes ricinus*; little is understood about the role that spleen-intact human populations and silent carriage of *B. divergens* might play in terms of risk to the blood supply.<sup>64</sup>

Greater awareness and scrutiny, together with molecular and biologic characterization of patient isolates of *Babesia* parasites, have yielded a more complex picture than historically thought, one still being elucidated. The predominant cause of human babesiosis in the United States—*B. microti*—has been denominated a diverse species complex composed of three distinct clades with identical morphology, but partly distinct vertebrate hosts and potentially differing levels of pathogenicity for humans.<sup>70</sup> Also in the United States, some, but not all, of the isolates from the Pacific Northwest have been grouped as a new species, *Babesia duncani*, that has been found in humans, dogs, and wildlife in the Western United States.<sup>71</sup> *Babesia* isolates from human cases in Missouri and Kentucky<sup>67,68</sup> and also from rabbits on Nantucket Island<sup>72</sup> that were originally attributed to *B. divergens* are now considered to be distinct from the agent of most human babesiosis in Europe.<sup>73</sup> These isolates, along with another from an asplenic man whose parasitemia surpassed 40%, are considered “*divergens*-like” on the basis of morphologic, serologic, and phylogenetic analysis.<sup>74</sup> This means that at least three distinct species of *Babesia* protozoa are known to cause human illness in the United States. As underappreciated as the risk for *B. microti* might be, even less is known about the risk posed to human health and the blood supply by the newly described species and isolates.

In Europe a similar picture is emerging. Although most cases of human babesiosis are attributed to *B. divergens*, a new

variant—designated EU1—has been isolated from asplenic patients in Italy and Austria. A near-identical isolate (EU3) was found in an asplenic German patient. Both organisms are phylogenetically closest to *B. divergens* and *B. odocoilei* (a parasite of white-tailed deer).<sup>75,76</sup> Also, the mounting entomologic<sup>77</sup> and serologic<sup>78</sup> evidence for human cases of *B. microti* in Europe has been confirmed by the first documented autochthonous case of human babesiosis in Europe caused by this agent, likely through blood transfusion.<sup>79</sup>

Finally, in East Asia, the first case of autochthonous human babesiosis in Japan has recently been confirmed, a result of transfusion-acquired *B. microti*-like infection.<sup>80</sup> Asymptomatic, *B. microti*-like infection has been documented in Taiwan.<sup>81</sup> The first case of human babesiosis has been reported from Korea, with the isolated parasite designated KO1 and appearing to be related to *Babesia* species that infect sheep.<sup>82</sup>

The two most salient features of *B. microti* infection contributing to transfusion risk are the probability that most infections are clinically silent<sup>83</sup> and the ability of immunocompetent persons to carry infection for lengthy periods.<sup>84</sup> There is no certain measure of what proportion of infections are asymptomatic, but seropositivity rates of blood donors or the general population tend to be orders of magnitude higher than the rates of symptomatic cases reported to public health departments. For example, the highest reported incidence of babesiosis in any part of Connecticut in 2002 was 62.7 cases per 100,000 population (0.06 %) in the town of Waterford.<sup>85</sup> However, a survey of blood donors in the region of the state of highest endemicity (southeast corner) indicated a point prevalence more than 20 times higher, at 1.4%.<sup>86</sup> Of 19 seropositive donors, 10 were confirmed parasitemic by PCR. Early research confirmed the ability of the parasite to persist for several months in untreated individuals.<sup>84</sup> In in-vitro studies, the parasite survives storage at 4°C and remains infective for up to 21 days,<sup>87</sup> and one case of transfusion transmission occurred after the storage of blood for 35 days.<sup>88</sup> The implications of long-term carriage of *B. microti* are amply demonstrated in a case in Minnesota, where an asymptomatic donor infected recipients from each of four donations over a 6-month period.<sup>89</sup>

In the United States, an estimated 60 transfusion cases of *Babesia* infection have occurred since the first report in 1980, with an estimate that 10 cases occur annually, many unpublished.<sup>90</sup> Most cases of transfusion transmission have involved RBCs, although transmission through platelet concentrates has also been reported.<sup>91</sup> In transfusion cases, symptoms typically develop 2 to 8 weeks following transfusion, and include fever, hemolytic anemia, and thrombocytopenia.<sup>92</sup> Transfusion transmission of *B. microti* has been documented once each in Canada,<sup>93</sup> Germany,<sup>79</sup> and Japan,<sup>94</sup> the only countries besides the United States to report transfusion cases of *Babesia*. Only the cases in Germany and Japan reflect autochthonous transmission, however. In Canada the donor acquired infection while on holiday in the United States. In the Pacific Northwest, the variant designated WA-1, since named *B. duncani*, has also been implicated in two cases of transfusion transmission.<sup>95</sup>



Few studies have directly measured the risk for transfusion transmission of *Babesia* parasites. Given the absence of symptoms in healthy individuals, studying paired donor-recipient specimens is the most secure way of estimating the risk in areas of endemicity. One study estimated a risk of 1 in 617 units (0.17%) of RBCs might be at risk for transmitting *B. microti* in the state of Connecticut, with the risk from platelets estimated as zero.<sup>96</sup> In another Connecticut study, analysis of paired donor-recipient specimens involving chronically transfused patients indicated one possible *B. microti* seroconversion in nearly 2000 evaluable transfusions.<sup>97</sup> Infectivity of different blood components is not easy to determine given that the likelihood of transmitting infection to and detecting clinical infection in the recipient are both likely to depend on the dose of parasites, the length of storage, and the recipient's immune status. In the aforementioned case in Minnesota, one of three recipients of platelets became infected with *B. microti*, while all three surviving recipients of red cells became infected.<sup>89</sup> In another case, of six individuals transfused with RBCs from an asymptomatic donor, two neonates and one elderly adult became infected, while two other neonates and a child remained aparasitemic.<sup>98</sup> Look-back studies in Connecticut found that 26% of recipients of RBC units became infected, compared to only 11% of those receiving platelet products.<sup>99</sup>

Traditional diagnosis of *Babesia* infection relies on microscopic examination of Giemsa-stained blood films. This method, however, requires an experienced microscopist, and a high enough index of clinical suspicion to perform the examination. Immunofluorescent assay diagnosis is both sensitive and specific for measuring exposure, but is too time consuming for mass screening, and involves subjectivity on the reader's part. Inoculation of blood from suspected human cases into laboratory animals offers both high sensitivity and specificity, but its utility is limited to research investigation. Real-time PCR appears to be up to three times as sensitive as conventional nested PCR for donors with variable serologic status.<sup>100</sup>

Human babesiosis has historically been treated with clindamycin and quinine (CQ), but atovaquone with azithromycin has emerged as the first-line recommendation in the United States because of fewer collateral effects.<sup>101</sup> Treatment with CQ continues to be recommended for severe cases, although partial or whole blood exchange transfusion might be performed in such cases.<sup>101,102</sup>

In the absence of a licensed screening assay, prevention of transfusion-transmitted babesiosis relies exclusively on asking donors if they have ever had babesiosis. This strategy is insufficient because only donors whose *Babesia* infection was patent and properly diagnosed will self-report a risk. Because the majority of *B. microti* infections are silent or remain undiagnosed,<sup>103</sup> parasitemic donors can escape detection. Seasonal deferral in areas of endemicity would adversely impact the blood supply, and would not address the issues of nonresidents who travel to *Babesia*-endemic areas<sup>93</sup> nor the transfer of blood components from areas where the infection is endemic to areas where it is not.

The predictive value of self-reported exposure to ticks in relation to serologic status has proven low in Wisconsin and Connecticut donors.<sup>104</sup>

As with other protozoan infections, pathogen inactivation methods hold promise in reducing the risk from transfusion-transmitted babesiosis. A range of photochemical treatments currently under development indicate reductions by 4 to 5 logs in plasma<sup>105</sup> and platelets.<sup>106</sup> Electrophilic agents that disrupt nucleic acid replication have shown potency in inactivating *B. microti* by bio-assay (hamster inoculation) and by PCR.<sup>107</sup>

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## Leishmaniasis

*Leishmania* species are a large group of protozoan parasites with broad distribution worldwide.<sup>108</sup> *Leishmania* parasites are transmitted in nature by the bite of a female phlebotomine sandfly, but can also be transmitted via blood components and, rarely, congenitally or sexually.<sup>109</sup> Clinical manifestations can vary widely, ranging from asymptomatic infection to severe illness with visceral, cutaneous, or mucosal involvement. The visceral form (kala-azar) is characterized by fever, wasting, hepatosplenomegaly, and pancytopenia; if untreated it is usually lethal.<sup>110</sup> Cutaneous forms involve progressive skin lesions that become ulcerative, sometimes with mucosal involvement. Although *Leishmania* species are endemic in about 88 countries, the public health burden is hardly uniform. More than 90% of visceral cases appear in Bangladesh, Brazil, India, Nepal, and Sudan, and about 90% of cutaneous leishmaniasis occurs in Afghanistan, Brazil, Peru, Iran, Saudi Arabia, and Syria.<sup>111</sup> The World Health Organization (WHO) estimates that 2 million new infections occur annually—about 1.5 million are cutaneous leishmaniasis (CL), and half a million are visceral leishmaniasis (VL); overall, as many as 12 million people worldwide are infected.<sup>111</sup> The distribution is expanding because of myriad factors that include ecological disturbances, growing urbanization in developing countries, and the growing numbers of people immunocompromised by human immunodeficiency virus (HIV) infection or other reasons.<sup>109</sup> The United States is home to both phlebotomine sandflies and *Leishmania* parasites, but autochthonous transmission is limited to infrequent and isolated outbreaks.<sup>112</sup>

The infective form of *Leishmania* parasites in the sandfly is the flagellated promastigote; in humans and other vertebrate hosts the parasite takes the form of an oval amastigote that is typically found in phagocytic vacuoles of macrophages and other mononuclear phagocytes.<sup>113</sup> Diagnostic methods include microscopic visualization of amastigotes in the tissue aspirates or biopsies from spleen, marrow, or lymph nodes, or in the peripheral blood buffy coat. The duration of parasite circulation in the blood can vary across the >20 *Leishmania* species, but in any case rarely lasts more than 1 year.<sup>114</sup> The immunologic response differs according to the clinical syndrome, with VL leading to a stronger and more enduring antibody production than CL.<sup>114</sup> The conventional treatment of leishmaniasis has been pentavalent antimony, but

new strategies include lipid formulations of amphotericin B, injectable paromomycin, and miltefosine.<sup>115</sup>

The risk for transfusion transmission of *Leishmania* may be underappreciated and its occurrence underreported, with 10 probable or confirmed cases having been reported in the literature<sup>116</sup> over the past 60 years. Half of these cases were in infants, and nine of 10 were in children 6 years of age or younger.<sup>108</sup> The average incubation period was over 7 months, with fever and hepatosplenomegaly being the most common symptoms.<sup>108</sup> Studies from areas of endemicity, as well as studies of US military personnel returning from operations in southwest Asia, highlight the risk. Viscerotropic *L. donovani* and *L. tropica* isolated from US armed service personnel have been shown to survive at least 25 days in Whole Blood or RBCs stored at 4°C, at least 5 days in platelet concentrates stored at 24°C, and at least 35 days in glycerolized RBCs frozen at -70°C. Further, *L. tropica*-spiked Whole Blood stored at 4°C for 30 days retained infectivity to healthy mice. Fresh frozen plasma did not support parasite survival.<sup>117</sup>

Because transfusion transmission would likely be mistaken for sandfly transmission in *Leishmania*-endemic areas, and because infection may remain subpatent in immunocompetent individuals, assessing the risk for transmission in blood is a challenge. The few studies involving blood donors indicate nonnegligible rates of detectable parasitemia. In one study in southern France, 76 of 565 blood donors were seropositive by Western blot, and direct detection via kinetoplast DNA amplification and/or direct culture from buffy coat samples were positive in 16 donors. The investigators concluded that *L. infantum* circulates intermittently and at low density, and that serologic screening of donors would be insufficiently specific to maintain blood availability.<sup>118</sup> Another study in the Balearic Islands of Spain found similar results. From 656 healthy blood donors, 2.4% to 7.6% were seropositive by different methods, and 22% of a subset of donors yielded positive cultures. Further, nine of 18 donors who were positive by nested PCR remained positive 12 months later.<sup>119</sup> Numbers of this magnitude suggest that the actual transfusion risk might be quite a bit higher than indicated by published reports, which represent cases detected in largely immunosuppressed individuals.

The options available to blood banks to prevent distribution of *Leishmania*-contaminated blood are limited. Targeted donor exclusion is practiced selectively in the United States, specifically relating to the risk of armed services personnel and other travelers to theaters of war harboring *Leishmania* species upon return. To prevent this, AABB established a 12-month deferral during Operation Desert Storm in Iraq in the early 1990s, and again more recently.<sup>116</sup> Given the broad distribution of *Leishmania* across 88 countries, travel-based deferrals might prejudice blood availability if strictly applied, while further lengthening and complicating the donor health history questionnaire. Because serologic status correlates poorly with asymptomatic infection,<sup>120,121</sup> antibody tests hold little promise for donor screening. Photochemical inactivation of different *Leishmania* species has shown 4-log reduction of amastigote and promastigote forms

in apheresis platelets,<sup>122</sup> and another study demonstrated 5- to 6-log reductions in both platelets and plasma.<sup>123</sup> Early results of another method using phototreatment alone shows promise in red cells.<sup>124</sup> Further, filtration of leukocytes at both the point of collection and the bedside has been shown to dramatically lower free and intracellular *Leishmania* parasites.<sup>125</sup>

## Toxoplasmosis

*Toxoplasma gondii* is an obligate, intracellular protozoan that can grow in any mammalian or avian organs or tissues.<sup>113</sup> Felines (the definitive host) become infected in nature by consuming intermediate rodent hosts with infective *Toxoplasma* cysts in the brain or skeletal muscle. The parasite is ubiquitous in nature and can infect a wide variety of animals, including sheep, cattle, and pigs, developing into cysts that remain infective for years. Human infection can occur through many modes of transmission. Most commonly, they include consumption of raw or undercooked meat containing *Toxoplasma* cysts, and accidental ingestion of *T. gondii* oocysts from soil or cat litter contaminated with excreted oocysts. Water is increasingly implicated as a vehicle for transmission.<sup>126</sup> There are rare reports of infection acquired via solid-organ transplantation and blood transfusion.<sup>113</sup> Finally, congenital transmission can occur when the mother acquires primary infection during pregnancy, but the probability of transmission to the fetus (and clinical outcome) depends on the timing of infection.<sup>127</sup>

Up to one-third of the world's population is estimated to be infected with *T. gondii*. About 90% of primary infections are subclinical, and appear to be lifelong. Illness in those with patent infections is usually non-specific and self-limiting, typically involving fever and isolated swollen lymph nodes. More rarely, infection might lead to myocarditis, pneumonitis, hepatitis, or encephalitis.<sup>128</sup> Toxoplasmosis is more frequently severe in immunocompromised hosts, and it has emerged as a common opportunistic infection of persons with AIDS.<sup>129</sup> Estimates of seroprevalence across countries vary from less than 10% to greater than 90%, and the acquisition of infection depends on an array of local and household factors that include hygiene and sanitation, source and preparation of food, exposure to felines, and climatic factors influencing the survivability of oocysts in nature.<sup>128</sup> In the United States, the National Health and Nutrition Examination Survey (NHANES) from 1988 to 1994 indicated an age-adjusted seroprevalence of 22.5% for those above 12 years of age.<sup>129</sup> The subsequent NHANES (1999-2004), also drawn from a nationally representative sample, demonstrated a decline in seroprevalence greater than one-third for US-born residents between 12 and 49 years of age. Notably, foreign-born residents living in the United States were three times as likely as US-born residents to be antibody-positive, 24.8% vs 8.2%, respectively.<sup>130</sup>

Transfusion transmission of toxoplasmosis was reported nearly 40 years ago, in a case where patients with acute leukemia were transfused with leukocytes from donors with chronic

myelogenous leukemia.<sup>131</sup> Although few reports of transfusion transmission are recorded in the literature, the risk has been well documented in recipients of cardiac transplant,<sup>128</sup> bone marrow transplant,<sup>132</sup> and more recently, small-bowel and other solid-organ transplantation.<sup>133</sup> Limited seroprevalence studies have been conducted in healthy blood donors, indicating a broad range of antibody prevalence: 7.4% in Durango, Mexico<sup>134</sup>; 9.6% in northeast Thailand<sup>135</sup>; 20% in Turkey<sup>136</sup> and southern India<sup>137</sup>; and 75% in northeastern Brazil.<sup>138</sup> Antibody presence is long-lived and does not necessarily denote infectivity. Little information is available on the long-term kinetics of antibody development and patent parasitemia. Advances in diagnostic methods include nested PCR<sup>139</sup> and rapid, real-time PCR,<sup>140</sup> but these assays are used principally for diagnosis of congenital and ocular toxoplasmosis and with immunocompromised patients.<sup>128</sup> Parasite isolation or detection by PCR is rarely useful in immunocompetent patients,<sup>141</sup> indicating low probability of detectable parasitemia. One study in Brazil performed real-time PCR on buffy coat samples from individuals classified by serology as chronically infected, and found 3.6% (4 of 110) were positive.<sup>140</sup> The mechanisms regulating the persistence of *T. gondii* infection and the conversion from quiescent to active infection are still being elucidated.<sup>142</sup> Although PCR has been established as effective at diagnosing ocular toxoplasmosis in immunocompetent patients,<sup>128</sup> the presence of low-grade or intermittent parasitemia in healthy individuals remains little studied. However, the ability of the parasite to survive 50 days at 4°C,<sup>131</sup> and the isolated reports of transfusion transmission in the literature, both establish an element of risk.

Prevention of transfusion-transmitted toxoplasmosis is not feasible with either donor exclusion or serologic screening. In most places, discarding units from seropositive donors would heavily prejudice blood availability, with unclear indications that positive antibody status of the donor implies risk for parasitemia. In high-prevalence countries, many blood recipients are likely to have been previously exposed. In an immunocompetent recipient, transfusion transmission is likely to go undetected. Given the parasite's ability to readily invade and replicate in leukocytes,<sup>143,144</sup> leukocyte filtration might diminish the risk in similar fashion as with cytomegalovirus. Whether inactivation treatments being evaluated for other protozoa might be of use for *Toxoplasma* remains unexplored.

## Microfilariasis

Filarial worms are arthropod-borne macroparasites that can be caused by a number of different organisms. *Wuchereria bancrofti* and *Brugia spp.* cause lymphatic filariasis; *Loa loa*, *Onchocerca volvulus*, and *Mansonella streptocerca* cause nonlymphatic, subcutaneous filariasis; and *M. ozzardi* and *M. perstans* cause nonlymphatic infections of different body cavities and are typically asymptomatic or mild.<sup>113</sup> The filariases occur in more than 80 countries, and the health and socioeconomic burden from

lymphatic filariasis and *O. volvulus* are particularly severe, with an estimated prevalence of 120 million and 37 million infections, respectively.<sup>145</sup> These organisms share similar life cycles and are all transmitted by hematophagous arthropods. In each, adult female worms produce larvae called microfilaria, which for most species circulate in the bloodstream, sometimes with periodicity timed to their primary insect vector's feeding habits.<sup>113</sup> The microfilaria are the infective form for insects, but when transmitted by transfusion are incapable of propagating further.<sup>146</sup>

The lymphatic forms of filariasis, which cause elephantiasis, have been targeted by WHO as potentially eradicable, primarily by eliminating the human reservoir of microfilaria that infect the mosquito vectors through repeated mass administration of curative drugs.<sup>147,148</sup> Results from low- to moderate-prevalence areas in Egypt<sup>149</sup> and from moderate- to high-prevalence parts of Papua New Guinea<sup>150</sup> indicate dramatic success in lowering human infections and even, surprisingly, reversing the pathology associated with infection.

There is little published information on the risk for transfusion transmission of filariasis. In most filariasis-endemic areas, the risk for vector-acquired infection would be orders of magnitude greater than that from transfusion for the average individual, given the relative degree of exposure to infective insect vectors and the limited rate of blood transfusion. However, limited studies from Nigeria have shown prevalence of microfilaria of 3.5% with *Loa loa*<sup>151</sup>; 15.6% with *M. perstans* and 1.3% with *L. loa*<sup>152</sup>; and 1.3% with unspecified microfilaria.<sup>153</sup> There has been at least one report of an American blood donor being found with microfilaria.<sup>146</sup> Isolated case reports of transfusion transmission exist from Italy<sup>154</sup> and India,<sup>155</sup> in both cases indicating that the outcome in blood recipients might often be no more severe than mild allergic reaction in response to microfilarial antigens.

The microfilaria of both *W. bancrofti*<sup>156</sup> and *L. loa*<sup>157</sup> survive routine storage conditions for blood. Those from *M. ozzardi*<sup>158</sup> and from *B. malayi* and *W. bancrofti*<sup>159</sup> have been successfully recovered following cryopreservation in research laboratories. One article from Spain reports an evaluation of alternative strategies for screening for *L. loa* microfilaria in donors from Central and West Africa.<sup>160</sup> This example is the exception, however, as most countries appear to consider the risk to blood recipients too small to merit donor or component screening.

## Conclusion

The risk for transfusion transmission of parasites in the United States is not high, but might well be greater than the prevailing estimated risks of one per million units. Until the recent licensure of a screening assay, it appears likely that hundreds of individuals have donated annually while infected with *T. cruzi*, the result of demographic changes in the United States and its donor population. Even assuming low-end estimates of infectivity, that implies dozens of cases of transfusion-transmission annually. The clinical impact of such events might be

uncertain, but at least in immunocompromised blood recipients the risk is serious.

Perceived risk for *Babesia* species in blood might not be widespread, but the combination of asymptomatic infection in healthy donors, the lack of a licensed screening assay, and the mobility of both people and blood components implies risks that are higher and geographically less circumscribed than appreciated.

Silent infection in semi-immune donors contributes to risk for malaria transmission in blood components, but recent years have seen an average of one case per year. Serologic or direct detection assays can both contribute to enhanced safety against these and other parasitic agents. Broadly effective methods such as pathogen removal or inactivation also appear promising.

## Disclaimer

The author has disclosed no conflict of interest.

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Bacterial contamination of blood components is a persistent but often overlooked problem in transfusion medicine. Before recent developments in skin preparation, diversion, and testing for bacterial contamination, the incidence of platelet bacterial contamination was approximately 1 in 1000.<sup>1</sup> Although recent public attention has focused on transfusion-transmitted viral infection, improved methods of screening through donor questioning and testing have greatly reduced the transmission of hepatitis viruses and retroviruses. Given the reduction of viral transmission via allogeneic blood, the risk of bacterial contamination has emerged as the greatest residual threat of transfusion-transmitted disease.

This chapter provides a quick overview of risks associated with blood components. The main focus is on approaches to minimize or eliminate the risk of bacterial contamination.

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**Transfusion-Transmitted Bacterial Infection of Red Blood Cells**

Sepsis associated with the transfusion of bacterially contaminated Red Blood Cell (RBC) components is generally regarded as a very rare event. From 1995 to 2004, 25 fatalities thought to be secondary to contamination of Whole Blood or RBC units were reported to the US Food and Drug Administration (FDA) (Fig 49-1).<sup>2</sup> The risk of death from a bacterially contaminated RBC transfusion in the United States has been estimated to be 0.13 per million.<sup>3</sup> Reports from New Zealand have indicated a *Yersinia enterocolitica* transfusion-transmitted incidence rate of 1 in 65,000, with a fatality rate of 1 in 104,000 RBC units transfused.<sup>4</sup> Unrecognized cases, underreporting, and regional variation may account for observed differences in the incidence.

Passive reporting studies from the United States,<sup>3</sup> France,<sup>5</sup> and United Kingdom (UK)<sup>6</sup> of bacterially contaminated RBCs that caused symptoms of infection show a relative paucity of

*Yersinia* cases (Table 49-1). Of the reported deaths, one was caused by a coagulase-negative *Staphylococcus* strain and seven were caused by a variety of gram-negative organisms. These organisms are all capable of growth at 1 to 6°C. Sepsis associated with the transfusion of gram-negative bacterially contaminated RBCs is typically severe and rapid in onset. Patients frequently develop high fevers (temperatures as high as 109°F have been observed) and chills during or immediately following transfusion. From 1987 to 1996, 20 cases of *Yersinia*-infected RBC units in 14 states were reported to the Centers for Disease Control and Prevention (CDC).<sup>8</sup> Twelve of the 20 recipients died in 37 days or less following transfusion. The median time from transfusion to death was 25 hours. Of the seven who developed disseminated intravascular coagulation, six died.

**Allogeneic RBC Units**

Asymptomatic donors with transient *Yersinia* bacteremia are presumed to be the source of contamination. Implicated donors are typically found to have infection elevated antibody titers [immunoglobulin M (IgM) or IgG] to *Y. enterocolitica*, implying a recent infection.<sup>8-10</sup> The possibility of contamination of RBC components is directly related to their storage time; most cases of *Yersinia* contamination of RBC units occur after storage for 25 days.<sup>11</sup>

*Serratia marcescens* was linked to an outbreak caused by contamination of RBCs in Denmark and Sweden.<sup>12,13</sup> The contamination was thought to involve the manufacturing process, because the sterile bag sets were autoclaved and put in a clean but not sterile outer plastic package. It was thought that *S. marcescens* present in the dust in the factory contaminated the outside of the containers, and in the presence of moisture and a nutrient (the plasticizer diethylhexylphthalate), the bacteria proliferated and somehow gained entry into the bag.<sup>14</sup>

Prospective bacterial cultures of Whole Blood or RBC units, however, have shown a much higher incidence of bacterial contamination (2 to 4 per 1000 units). The organisms commonly cultured are *Staphylococcus* or *Propionibacterium* spp., which generally proliferate poorly during storage at 1 to 6°C.

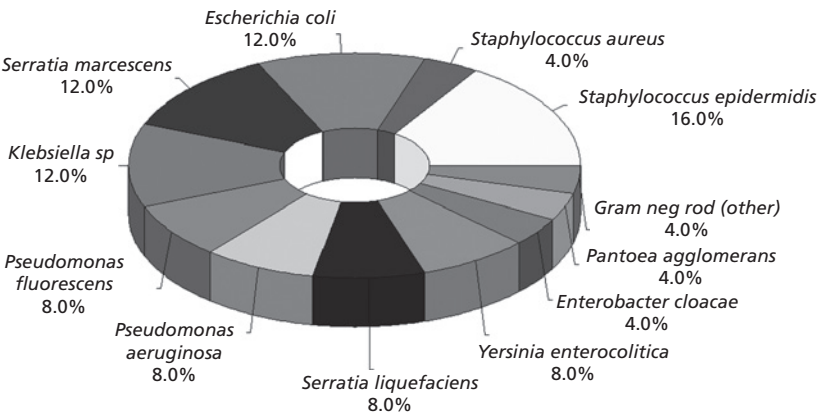


**Figure 49-1.** Transfusion fatalities by organism in RBC units or whole blood, as reported to the United States Food and Drug Administration from 1995-2004 10 yrs, 25 cases (data from Niu et al<sup>2</sup>).

**Table 49-1.** Summary of Organisms from RBC Transfusions Identified in the BACON, SHOT, and BACTHEM Studies\*

| Organism                                | United States<br>BACON | United Kingdom<br>SHOT | France BACTHEM<br>(29 isolates, 25<br>implicated units) | Total         |
|-----------------------------------------|------------------------|------------------------|---------------------------------------------------------|---------------|
| <b>Gram positive</b>                    |                        |                        |                                                         |               |
| Coagulase-negative <i>Staphylococci</i> | 2(1)                   | 2                      | 3                                                       | 7(1)          |
| <i>Streptococcus</i> spp.               |                        |                        | 4 <sup>†</sup>                                          | 4             |
| <i>Staphylococcus aureus</i>            |                        |                        | 2 <sup>†</sup>                                          | 2             |
| <i>Enterococcus faecalis</i>            |                        |                        | 1 <sup>§</sup>                                          | 1             |
| <i>Bacillus cereus</i>                  |                        |                        | 2                                                       | 2             |
| <i>Propionibacterium acnes</i>          |                        |                        | 1                                                       | 1             |
| Subtotal                                | 2 (1 or 50%)           | 2 (0 or 0%)            | 13 (0 or 0%)                                            | 17 (1 or 6%)  |
| <b>Gram negative</b>                    |                        |                        |                                                         |               |
| <i>Serratia liquefaciens</i>            | 2(2)                   | 1                      | 2(1)                                                    | 5(3)          |
| <i>Serratia marcescens</i>              | 1                      |                        |                                                         | 1             |
| <i>Yersinia enterocolitica</i>          |                        | 1(1)                   | 1                                                       | 2(1)          |
| <i>Enterobacter</i> sp.                 |                        |                        | 1(1)                                                    | 1(1)          |
| <i>Acineterbacter</i> sp.               |                        |                        | 5(1) <sup>‡</sup>                                       | 5(1)          |
| <i>Pseudomonas</i> sp.                  |                        |                        | 2(1)                                                    | 2(1)          |
| <i>Escherichia coli</i>                 |                        |                        | 3 <sup>§</sup>                                          | 3             |
| <i>Klebsiella pneumoniae</i>            |                        |                        | 1                                                       | 1             |
| <i>Proteus mirabilis</i>                |                        |                        | 1                                                       | 1             |
| Subtotal                                | 3 (2 or 67%)           | 2 (1 or 50%)           | 16 (4 or 25%)                                           | 21 (7 or 33%) |
| <b>Total</b>                            | 5 (3 or 60%)           | 4 (1 or 25%)           | 29 (4 or 14%)                                           | 38 (8 or 21%) |

\*Numbers of fatalities and the percent of the total are reported in parentheses.  
<sup>†</sup>In two cases, two isolates of *Streptococcus* sp. were isolated from the implicated bag.  
<sup>‡</sup>In one case, *Staphylococcus aureus* and *Acineterbacter baumannii* were both isolated from the implicated bag.  
<sup>§</sup>In one case, *Enterococcus faecalis* and *Escherichia coli* were both isolated from the implicated bag.  
Used with permission from Brecher and Hay.<sup>7</sup>

Autologous RBC Units

Although autologous blood is generally considered a “safer” blood component, there have been at least six reported cases of bacterial contamination of autologous RBC units, five

from *Y. enterocolitica*<sup>15-18</sup> (and personal communication, Susan Cookson, CDC, September 23, 1996) and one from *Serratia liquefaciens*.<sup>19</sup> Fortunately, all recipients survived, possibly because of preformed immunity. Upon retrospective

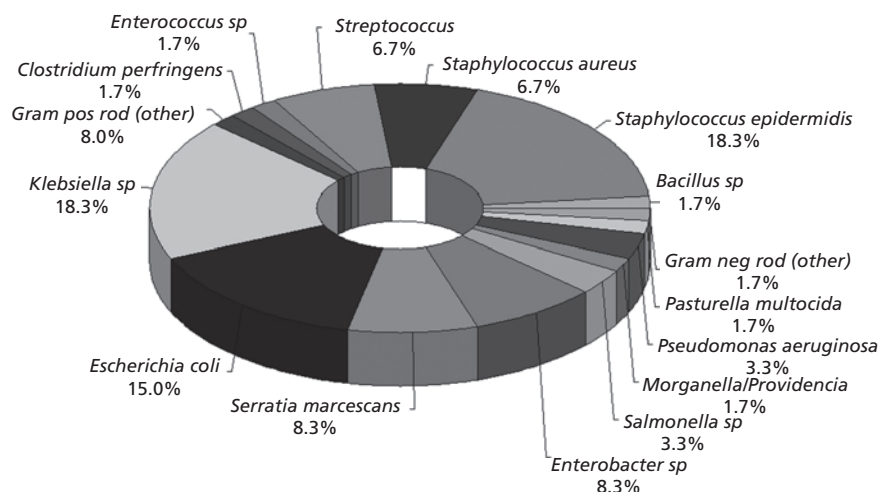


**Figure 49-2.** Transfusion fatalities by organism in platelets, as reported to the United States Food and Drug Administration from 1995-2004 10 yrs, 60 cases (data from Niu et al<sup>2</sup>).

questioning, all patients infected by *Yersinia* recalled gastrointestinal symptoms in the days before donation. In the case of *Serratia* contamination, the patient's infected toe ulcer was presumed to be the source.

### Transfusion-Transmitted Bacterial Infection of Plasma, Cryoprecipitate, and Derivatives

Cell-free products, such as plasma and cryoprecipitate, are stored in the frozen state and thus are rarely associated with significant contamination. However, *Pseudomonas cepacia* and *P. aeruginosa* have been cultured from cryoprecipitate and plasma thawed in contaminated waterbaths.<sup>20,21</sup>

Human serum albumin is a good culture medium and preserves viability of contaminants. The heating step (60°C for 10 hours) in the manufacturing of albumin is performed to inactivate certain viruses, not to ensure bacterial sterility.<sup>22,23</sup> This would require autoclaving (superheated under pressure), which would cause albumin to denature. On occasion, specific lots of albumin product have been found to be contaminated with bacteria, typically *Pseudomonas* spp.<sup>24</sup> These lots have produced endotoxic shock, transient bacteremias, and febrile reactions in recipients. Two patients in different hospitals developed *Enterobacter cloacae* septicemia after receiving albumin.<sup>24,25</sup> Cultures of unopened product grew *Stenotrophomonas multophilus* and *Enterococcus gallinarum* in addition to *E. cloacae*. This resulted in a worldwide recall of certain lots of 5%, and 25% albumin. It is suspected that cracks in the glass bottles were responsible for the contamination. Manufacturing problems, therefore, are a source of bacterial risk from these derivatives.

### Transfusion-Transmitted Bacterial Infection of Platelets

#### Source of Contamination

Skin commensal organisms such as *Staphylococcus epidermidis* and *Bacillus cereus* are the organisms most often implicated in platelet bacterial contamination.<sup>26,27</sup> These organisms typically do not grow at 0 to 6°C, but survive and multiply readily at 20 to 24°C, the storage temperature of platelets. Fatalities caused by platelet contamination tend to be predominantly gram-negative organisms (Fig 49-2). Potential sources of these organisms include 1) donor-related transient bacteremia; 2) incomplete disinfection or skin core removal by the collection needle; and 3) contamination of the collection bag, tubing, or anticoagulant. In some cases, retrograde flow from vacuum tubes used in collection or even the recipient have been implicated as a source of contamination.<sup>28,29</sup>

#### Clinical Presentation

The clinical sequelae resulting from transfusion of bacterially contaminated platelets range from asymptomatic to mild fever (which may be indistinguishable from a nonhemolytic transfusion reaction) to acute sepsis, hypotension, and death. The clinical picture is much more varied and often less severe than that of patients infected by transfusion of bacterially contaminated RBCs.<sup>30</sup> Sepsis caused by transfusion of contaminated platelets is vastly underrecognized and underreported. Those patients who receive the greatest number of platelets (patients being treated with chemotherapy who are both thrombocytopenic and immunosuppressed) are those most at risk of sepsis (Table 49-2). In such patients fever can be readily

attributed to other infectious causes. Broad-spectrum antibiotics should be considered for any patient who develops fever within 6 hours of platelet transfusion.<sup>31,32</sup>

Incidence

Sepsis resulting from transfusion of bacterially contaminated platelets is the most common transfusion-transmitted disease. Platelets are stored at room temperature, 20 to 24°C, making them an excellent growth medium. Multiple aerobic-culture surveillance studies have demonstrated that 1 in 1000 to 3000 platelet units are bacterially contaminated (Table 49-3). Based on the fact that approximately 10 million platelet units are transfused every year in the United States,<sup>39</sup> it has been estimated that 2000 to 4000 bacterially contaminated platelets units are transfused every year. Despite the estimates of contamination, the actual septic transfusion reaction rates were much lower at approximately 1 in 25,000 platelet units (range 1 in 13,000 to 100,000).<sup>3,5,40</sup> Observations from hospitals actively pursuing platelet-related sepsis support these estimates. Johns Hopkins found a fatality rate of 1 in 17,000 for pooled platelet-rich plasma, whole-blood-derived (PRP-WBD) platelets

Table 49-2. Factors Affecting Outcome of Transfusion of Bacterially Contaminated Blood Components

- Virulence of the organism
- Immune status and general condition of the recipient
- Concentration and bolus dose of bacteria transfused
- Timely recognition and therapeutic intervention
- Intensity of patient monitoring—ie, inpatient vs outpatient
- Medicines the patient is receiving—ie, antibiotics

Used with permission from Krishan and Brecher.<sup>31</sup>

Table 49-3. Selected Culture Studies of Whole-Blood-Derived Platelet Concentrates (PC) and Apheresis Platelets (Aph)

| Country | Product | Sample volume (mLs) | Method     | Day of Sampling | "N"    | Initial Positive        | Confirmed Positive | Rate per 1000 Units | Ref                             |
|---------|---------|---------------------|------------|-----------------|--------|-------------------------|--------------------|---------------------|---------------------------------|
| Belgium | PC      | NS                  | BacT/Alert | NS              | 17675  | 294 (1.7%)              | 143 (0.81%)        | 8.1                 | Claeys et al <sup>33</sup>      |
|         | Aph     |                     |            |                 | 6885   | 98 (1.4%)               | 12(0.17%)*         | 1.7                 |                                 |
| Belgium | PC      | 2.5-5.0             | BacT/Alert | 0               | 7644   | 140 (1.8%) <sup>†</sup> | NS                 | 18                  | Schelstaete et al <sup>34</sup> |
|         | Aph     |                     |            |                 | 1949   | 34 (1.8%) <sup>†</sup>  |                    | 18                  |                                 |
| Brazil  | PC      | 2                   | BacT/Alert | 0               | 13,454 | 35 (0.26%)              | 21 (0.16%)         | 1.6                 | Wendel et al <sup>35</sup>      |
|         | Aph     |                     |            |                 | 3,553  | 21 (0.59%)              | 16 (0.45%)         | 4.5                 |                                 |
| US      | PC      | 5                   | Broth      | 6–12            | 4,995  | NS                      | 4 (0.08)           | 0.8                 | Leiby et al <sup>36</sup>       |
| US      | PC      | NS                  | Plate      | 4–5             | 23,390 | NS                      | 12 (0.05%)         | 0.5                 | Dykstra et al <sup>37</sup>     |
|         | Aph     |                     |            |                 | 5,147  | NS                      | 3 (0.06%)          | 0.6                 |                                 |
| Canada  | PC      | 2                   | Bactec     | 1               | 16,290 | NS                      | 4 (0.02%)          | 0.2                 | Blajchman et al <sup>38</sup>   |
|         |         |                     |            | 3               | 10,065 | NS                      | 7 (0.07%)          | 0.7                 |                                 |

\*Extrapolated from units available for retesting (125 PCs and 27 Aph).

<sup>†</sup>Based on the successful subculture of the culture bottle [originally 144 (1.9%) and 38 (2.0%), respectively].

and 1 in 61,000 for apheresis units.<sup>40</sup> The University Hospitals of Cleveland reported a fatality rate of 1 in 48,000 per PRP-WBD platelets.<sup>41</sup> However, it is widely suspected that platelet bacterial sepsis is frequently unrecognized and, thus, is underreported. From October 1, 1995 to September 30, 2004, there were 60 transfusion-transmitted fatalities caused by contamination of platelets reported to the FDA (2) (Tables 49-4 and 49-5).

Table 49-4. Fatalities from Platelet Transfusion in the United States from 1995 to 2004 (data from Nin et al<sup>2</sup>)

| Organisms                                       | Number of Deaths |
|-------------------------------------------------|------------------|
| <b>Gram-Positive (n=22)</b>                     |                  |
| <i>Staphylococcus epidermidis</i>               | 11               |
| <i>Staphylococcus aureus</i>                    | 4                |
| <i>Streptococcus</i> group G                    | 2                |
| <i>Clostridium perfringens</i>                  | 1                |
| <i>Streptococcus</i> group F / <i>Eikenella</i> | 1                |
| One unidentified gram-positive rod              | 1                |
| <b>Gram-Negative (n=38)</b>                     |                  |
| <i>Klebsiella pneumoniae</i>                    | 10               |
| <i>Escherichia coli</i>                         | 9                |
| <i>Serratia marcescans</i>                      | 5                |
| <i>Enterobacter aerogenes</i>                   | 2                |
| <i>Enterobacter cloacae</i>                     | 2                |
| <i>Pseudomonas aeruginosa</i>                   | 2                |
| <i>Salmonella</i> spp.                          | 2                |
| <i>Bacillus</i> spp.                            | 1                |
| <i>Enterobacter agglomerans</i>                 | 1                |
| <i>Klebsiella oxytoca</i>                       | 1                |
| <i>Morganella/Providencia</i>                   | 1                |
| <i>Pasturella multocida</i>                     | 1                |
| Two unidentified gram-negative rods             | 1                |

## Prevention

Both the College of American Pathologists (CAP) and AABB have instituted steps to require the detection of bacteria in platelet products. In December 2002, the CAP added a phase I item to the Laboratory Accreditation Checklist regarding the detection of bacteria.<sup>42</sup> The checklist item, TRM.44955, asked “Does the laboratory have a system to detect the presence of bacteria in platelet components?” The question was revised in December 2004 to ask “Does the laboratory have a validated system to detect the presence of bacteria in platelet components?” With this revision, the CAP ceased allowing swirling to be an acceptable method of detection, but still allows the use of surrogate testing methods such as pH or glucose measurements.

In March 2004, AABB instituted a new standard that requires blood banks or transfusion services to have steps in place to limit and detect bacterial contamination in all platelet products.<sup>43</sup> Since that time, blood centers and transfusion services have been implementing a variety of interventions to accomplish this goal.

The American Red Cross (ARC) began routine aerobic cultures using the BacT/ALERT (bioMérieux, Durham, NC) at that time and in their first 10 months of testing, 226 of 350,658 platelet collections initially tested positive.<sup>44</sup> Sixty-eight of these were confirmed positives for a rate of bacterial contamination of 1 in 5157. In the first two years of testing by the ARC, the confirmed positive rate was 1 in 5399 apheresis platelet units.<sup>45</sup> Despite universal testing of all platelet products, in the 2-year period from March 2004 through May 2006, the ARC reported 20 septic transfusion reactions caused by transfusion of

bacterially screened units. Of the 20 septic reactions, three were fatal and involved a *Staphylococcus* species. All of the units involved with septic fatalities and 13 of the units implicated in septic reactions were associated with platelets transfused on the fifth day after collection. If the units implicated in the septic transfusion reactions (which were culture negative) were considered to have tested as false-negative, the false-negative rate would be 1 in 74,807.<sup>46</sup>

The effects of the AABB standard are difficult to quantify. On the basis of 2006 platelet utilization data, in the United States in one year, approximately 2.8 million platelet units were transfused (1.5 million apheresis platelets and 1.3 million WBD platelets).<sup>39</sup> Using different fatality projections, anywhere from eight fatalities per year, based on the BaCon study,<sup>3</sup> up to 40 fatalities per year, based on a 1% fatality rate<sup>46</sup> would be expected. From 1995 to 2004, 60 deaths caused by bacterially contaminated platelets were reported to the FDA, averaging six deaths per year before the initiation of the standard.<sup>2</sup> Using the ARC data for 2004 through 2006 with additional data from Blood Systems, Inc. (1 probable septic transfusion reaction in 209,654 collections tested) and the New York Blood Center (no septic transfusion reactions in 150,241 collections tested), the passively reported septic reaction rate with platelets since testing is approximately 1 in 230,000.<sup>47</sup> This is compared to an approximate rate of 1 in 40,000 reported by ARC for a 10-month period in 2003.<sup>44</sup> This shows a decrease in the risk of septic reactions from platelets since the institution of the AABB standard, but these data are limited by virtue of being passively reported.<sup>47</sup>

**Table 49-5.** Summary of Organisms from Platelet Transfusions Identified in the BaCon, SHOT, and BACTHEM studies\*

| Organism                       | United States<br>BACON | United Kingdom<br>SHOT | France<br>BACTHEM    | Total                 |
|--------------------------------|------------------------|------------------------|----------------------|-----------------------|
| <b>Gram positive</b>           |                        |                        |                      |                       |
| <i>Bacillus cereus</i>         | 1                      | 4 (1)                  | 2                    | 7 (1)                 |
| Coagulase-negative             |                        |                        |                      |                       |
| <i>Staphylococci</i>           | 9                      | 8 (1)                  | 5                    | 20 (1)                |
| <i>Streptococcus</i> spp.      | 3 (1)                  | 2                      |                      | 5 (1)                 |
| <i>Staphylococcus aureus</i>   | 4                      | 3 (1)                  |                      | 6 (1)                 |
| <i>Propionibacterium acnes</i> |                        |                        | 3                    | 3                     |
| Subtotal                       | 17 (1 or 6%)           | 14 (3 or 21%)          | 10 (0 or 0%)         | 41 (4 or 10%)         |
| <b>Gram negative</b>           |                        |                        |                      |                       |
| <i>Klebsiella</i> spp.         |                        |                        | 2 (1)                | 2 (1)                 |
| <i>Serratia</i> spp.           | 2 (2)                  |                        | 1 (1)                | 3 (3)                 |
| <i>Escherichia coli</i>        | 5 (1)                  | 4 (2)                  | 1                    | 8 (2)                 |
| <i>Acinetobacter</i>           |                        |                        | 1                    | 1                     |
| <i>Enterobacter</i> spp.       | 2 (1)                  | 2 (1)                  | 1                    | 4 (2)                 |
| <i>Providencia rettgeri</i>    | 1 (1)                  |                        |                      | 1 (1)                 |
| <i>Yersinia enterocolitica</i> | 1                      |                        |                      | 1                     |
| Subtotal                       | 11 (5 or 45%)          | 3 (2 or 67%)           | 6 (2 or 33%)         | 20 (9 or 45%)         |
| <b>Total</b>                   | <b>28 (6 or 21%)</b>   | <b>17 (5 or 29%)</b>   | <b>16 (2 or 13%)</b> | <b>58 (13 or 22%)</b> |

\*Numbers of fatalities and the percent of the total are reported in parentheses. Used with permission from Brecher and Hay.<sup>7</sup>

Strategies to Reduce the Risk of Posttransfusion Sepsis

Several approaches to the reduction of risk of posttransfusion sepsis can be grouped into four major categories: bacterial avoidance, growth inhibition, detection, and elimination.

Bacterial Avoidance

Donor Screening

Histories of recent dental procedures, gastrointestinal or genitourinary procedure, and breast feeding (which can be associated with skin cracks) may all be associated with bacteremia and are cause to defer a potential donor. Unfortunately, asking donors about symptoms suggestive of infections are problematic. For example, 13% of donors have had gastrointestinal symptoms in the 30 days before donation.<sup>48</sup> In addition, retrospective questioning of donors implicated in *Yersinia* red cell sepsis showed that only half had any gastrointestinal symptoms in the preceding 30 days.<sup>8-10</sup> Therefore, donor questioning about gastrointestinal symptoms does not appear to be a specific predictor of *Yersinia* bacteremia.

Skin Preparation

Despite excellent technique, one cannot ensure a sterile venipuncture, because organisms harbored in sebaceous glands and hair follicles cannot be completely removed or killed, and skin fragments drawn up into the collection bag during the initial phase of donation can provide a source of infectious organisms.<sup>49,50</sup> Scarring or dimpling of the venipuncture site from prior donation has also been recognized as a risk factor for bacterial contamination, because these areas frequently are difficult to disinfect.<sup>51</sup> In one case, phlebotomy at a dimpled venipuncture site of an apheresis donor resulted in three episodes of platelet contamination with gram-positive organisms; sepsis occurred in four recipients of those platelets.

Iodine solutions have been shown to be the most effective in reducing the donor skin bacterial burden (Table 49-6). Skin of donors who are allergic to iodine is often cleansed with a chlorhexidine solution or double isopropyl alcohol skin

disinfection. In general, tincture of iodine, povidone iodine, or chlorhexidine are used for skin preparation. Table 49-7 summarizes the effectiveness of the most commonly used disinfection methods.<sup>53</sup>

Diversion

Diversion of the first few milliliters of whole blood from the primary container has been shown to reduce the amount of bacterial contamination from the skin. A study performed on 22,000 blood donations by the Red Cross in the Netherlands, in which the first 10 mL of donor blood were diverted from the primary bag, showed that 16 of the first 5-mL aliquots were bacterially contaminated, while only two of the second 5-mL aliquots were positive after culture.<sup>54</sup> A second study from the Netherlands compared the bacterial contamination rates of whole blood collections with and without the removal of the first 10 mL. The diversion of the first 10 mL showed a significant decrease in bacterial contamination (18,263 collections with 0.39% contamination without diversion compared with 7,115 collections with 0.21% contamination with diversion,  $p < 0.05$ ).<sup>55</sup>

It must be remembered that diversion is most effective at decreasing contamination with skin flora.<sup>56,57</sup> A majority of bacteria-related fatalities involve gram-negative organisms, which are not interdicted by diversion. Using the ARC data for collections between 2004 and 2006, it is possible to compare the true-positive rates with and without diversion. The authors report on the contamination rate of one-arm collections and two-arm collections.

Table 49-7. Percentage and Mean Count of Donors with Bacteria after Skin Disinfection

| CFU/Plate  | Povidone Iodine × 2 | IPA and Iodine | IPA × 2 Sponge | IPA × 2 Swab |
|------------|---------------------|----------------|----------------|--------------|
| 0          | 39%                 | 79%            | 0%             | 29%          |
| ≤ 10       | 57%                 | 93%            | 69%            | 50%          |
| ≤ 100      | 75%                 | 100%           | 86%            | 68%          |
| ≤ 1000     | 96%                 | 100%           | 97%            | 89%          |
| Mean count | 175                 | 3              | 161            | 237          |

Used with permission from Brecher and Hay.<sup>7</sup> (Data from McDonald et al.<sup>53</sup>)

Table 49-6. Percentage of Donors with Bacteria Growth after Skin Disinfection

| Bacterial Colonies per Plate        | Povidone Iodine | Isopropyl Alcohol and Iodine Tincture | Chlorhexidine Gluconate | Green Soap and Isopropyl Alcohol |
|-------------------------------------|-----------------|---------------------------------------|-------------------------|----------------------------------|
| 0                                   | 34-49%          | 63%                                   | 60%                     | 0%                               |
| 1-10                                | 35-43%          | 34%                                   | 25%                     | 17%                              |
| 11-100                              | 10-14%          | 2%                                    | 12%                     | 47%                              |
| > 100                               | 0-13%           | 1%                                    | 3%                      | 36%                              |
| p value compared to povidone iodine |                 | <0.001                                | >0.3                    | <0.001                           |

Used with permission from Goldman et al.<sup>52</sup>

The two-arm collection set-ups involved a diversion pouch that was located on the venous return line, not the inlet line. The one-arm collections utilized a correctly placed diversion pouch. Table 49-8 shows the decrease in the contamination rate of skin flora with the use of a correctly placed diversion pouch.<sup>45</sup>

### Apheresis Platelet vs Whole-Blood-Derived Platelet Concentrates

Therapeutic doses of platelets can be obtained from a single donor through an apheresis procedure or from whole blood donation. Four to six platelets concentrates from whole blood donations must be pooled to make a therapeutic dose; therefore, it would be expected that pooled platelets obtained from multiple donors would be at higher risk of bacterial contamination. From 1986 to 1998, Johns Hopkins Hospital increased the use of apheresis platelets from 51.7% to 99.4% and saw a threefold reduction in septic transfusion reaction involving platelets, from 1 in 4818 transfusions to 1 in 15,098 transfusions.<sup>58</sup>

A survey of AABB-accredited blood centers, hospital blood banks, and transfusion services (in total, 900 institutions) in late 2004 examined platelet usage, supply, and testing methods.<sup>59</sup> Between 2003 and 2004 there was an 11.3% reduction in the use of WBD platelets and 77.2% of platelets transfused by the institutions surveyed were apheresis platelets. Not only do apheresis platelets decrease the risk of exposing a recipient to a contaminated unit, the type of platelet used appears to affect the type of testing performed to detect bacteria in the United States. Over 90% of apheresis units were tested with culture, whereas most WBD platelets were tested using a variety of methods including staining with microscopic examination and the use of markers of increased metabolism such as pH and glucose. In practice, many institutions have felt that culturing of individual WBD platelets to be impractical.

A new system, Acrodose PL (Pall, Covina, CA) is now available for prestorage pooling of WBD platelets. The Acrodose system allows institutions to pool WBD platelets and then store them for the life of the individual platelets. By using prestorage pooling, the volume of the product is acceptable for culturing with either the BacT/ALERT or enhanced bacterial detection

system (eBDS, Pall, East Hills, NY). Platelets collected in CPD or CP2D collections bags, both leukocyte-reduced and non-leukocyte-reduced, can be pooled with the Acrodose system. This would allow users of PRP-WBPC to transition from surrogate testing to the more sensitive method of culturing.

Prepooled whole-blood-derived platelets prepared by the buffy coat method have been available in Europe since the early 1990s. Recently, Canada has been in the process of transitioning from the platelet-rich plasma method to the buffy coat method. The experience in Europe is that the confirmed positive culture rate of prepooled buffy coat platelets is equivalent to apheresis platelets.<sup>60</sup> This may be a result of the overnight incubation in the presence of white cells.

### Growth Inhibition

#### Optimizing Storage Time

In 1991, the Blood Products Advisory Committee (BPAC) of the FDA reviewed all cases of *Yersinia* sepsis from RBC units reported to either the FDA or the CDC during the late 1980s. At that time all reported cases of RBC-associated *Yersinia* sepsis in the United States had occurred in units older than 25 days. As a result of timing necessary for the bacteria to attain a lethal concentration, the BPAC proposed reducing the storage time of RBCs from 42 to 25 days. This recommendation was subsequently rejected for several reasons.

1. A questionnaire distributed at the time revealed that 20% of RBC units in stock at over 1500 blood banks and transfusion services were more than 28 days old.<sup>61,62</sup> Discarding such units would have severely compromised the nation's blood supply.
2. A shorter outdate would require recruitment of new donors. It was estimated that the addition of a quarter of million donations per year would be required to replace the loss resulting from shorter outdates. This would involve additional risk, because first-time donors are more likely to carry disease because their blood has not been repeatedly tested, as has the blood of repeat donors.
3. Units less than 25 days old can also cause sepsis, so that decreasing the allowable storage time would lessen the problem but not eliminate it.<sup>11,63,64</sup>
4. Older units are less likely to transmit viruses such as human immunodeficiency virus (HIV) and human T-cell lymphotropic virus types I and II.<sup>65,66</sup>

Longer platelet storage time has also been associated with increased probability of contamination. In 1983, in the United States platelet storage for WBD platelets was transiently approved for 7 days. This 7-day storage was based on acceptable in-vitro function, in-vivo recovery, and survival data. However, because of anecdotal reports of bacterial proliferation over the extended storage time, the shelf life was returned in 1986 to 5 days. Unfortunately, merely decreasing the shelf life did not eliminate the problem of bacterial contamination. It has been shown that even a moderate inoculate [10 to 50 colony-forming units (CFU/mL)] of certain bacteria such as *B. cereus* and *P. aeruginosa* in platelets has a minimal lag phase with a doubling time of 1 to 2 hours.<sup>67</sup> This can lead to a bacterial load of  $10^8$  in just 1 to

**Table 49-8.** Comparison of One- and Two-Arm Apheresis Platelet Collections

|                                               | Two-arm procedures                | One-arm procedures*              | OR (95% CI)   |
|-----------------------------------------------|-----------------------------------|----------------------------------|---------------|
| Rate of skin contaminants per $10^5$ cultures | 17.2 (106 of 617,595 collections) | 7.8 (30 of 386,611 collections)  | 2.2 (1.5-3.3) |
| Rate of nonskin organisms per $10^5$ cultures | 5.5 (10 of 617,595 collections)   | 4.1 (16 of 386,611 collections)  | 1.3 (0.7-2.4) |
| Rate of true positives per $10^5$ cultures    | 22.7 (140 of 617,595 collections) | 11.9 (46 of 386,611 collections) | 1.9 (1.4-2.7) |

\*The diversion pouch was in the correct place on the one-arm procedures only.<sup>45</sup>

2 days. The lowest safest limit of bacteria and the time to clinical symptoms are unknown. In one well-documented outbreak of *Salmonella choleraesuis* in seven patients linked to one common repeat donor with an occult chronic osteomyelitis, the time to the onset of illness ranged from 5 to 12 days (mean 8.6 days). In all cases, the platelet units were stored for less than 1 day.<sup>68</sup> In a similar situation, the CDC reported in 2006 a multistate outbreak of *Pseudomonas fluorescens* bloodstream infections. All cases could be traced to contaminated heparin flushes.<sup>69</sup> A total of 28 patients had delayed onset of *P. fluorescens* infections, ranging from 84 to 421 days after their last potential exposure.

Only by severely reducing the storage time of platelets to 1 to 2 days would one significantly impact the risk of bacterial overgrowth. However, operational changes affect the storage age of available platelets. With increased complexity of disease-marker testing, the availability of 1-day-old platelets has decreased. For example, in the United States in 1982 the mean age of distributed platelets was 1.6 days, in 1983 (after extension of the dating period to 5 days) it was 2.0 days, and in 1992 (after addition of increased laboratory testing) it was 2.5 days. In 1983, only 5% of issued platelets were greater than 3 days old. In 1992, just 10% were older than 3 days. But with the introduction of centralized testing by the ARC, the mean age of issued platelets increased 2.7 days with 20% older than 3 days.<sup>70</sup> With the addition of nucleic acid testing for HIV and hepatitis C virus (HCV), additional delays occurred. Not only can this decrease available shelf life of an already precariously limited supply of platelets, it can also decrease the availability of fresh platelets, which are the most hemostatic and the least likely to be bacterially contaminated.

### Optimizing Storage Temperature

Bacterial proliferation in red cells or whole blood is generally limited to a few gram-negative organisms capable of proliferation at 1 to 6°C (so-called psychrophilic organisms, most notably *Y. enterocolitica*, *Serratia* spp., and *Pseudomonas fluorescens*). Freezing red cells with glycerol would prevent the proliferation of bacteria but would be associated with significant decreased availability and increased cost.

Platelets are typically stored at 20 to 24°C, principally because of a potential concern of bacterial contamination and progressive decline in platelet function (storage lesion) if stored for longer periods at these temperatures. Recently, with the addition of an early culture, the use of 7 day platelets has resumed in many parts of the world (see the discussion of international practice and specifically the United States Post Approval Surveillance Study of Platelet Outcomes, Release Tested (PASSPORT) later in the chapter). Although storage of platelets at 4°C results in a significantly lower rate of bacterial growth, it also causes a temperature-induced activation of platelets and rapid decline in functional ability and in-vitro viability. Studies of cold-storage platelets with the addition of specific second-messenger stimulators have reported that platelets stored at 4°C were bacteriostatic and retained partial functional ability and viability compared with control platelets.<sup>71,72</sup> If a practical method for storing platelets at 4°C or in a frozen state were perfected, it would have the potential to reduce the risk of bacterial contamination of this blood component.

### Addition of Antibiotics

The addition of antibiotics to blood components has been considered but has not been adopted because of the added risk of an adverse drug reactions (trading one infrequent reaction for another) and the potential for the development of antibiotic resistance if small amounts of antibiotics were infused with every transfusion.<sup>73</sup>

### Bacteria Detection

A simple low-cost method to detect bacterial overgrowth would be a valuable tool for screening units before release from the blood bank. Several methods for bacterial detection are available and more are in development (Fig 49-3). Unlike viral contamination of blood components, which is detected from a sample obtained at the time of donation, bacterial contamination of blood components frequently requires time for the organisms to proliferate before being detectable. Therefore, knowledge of growth characteristics of bacteria in blood components must be considered for successful implementation of a detection strategy (Figs 49-4 and 49-5).

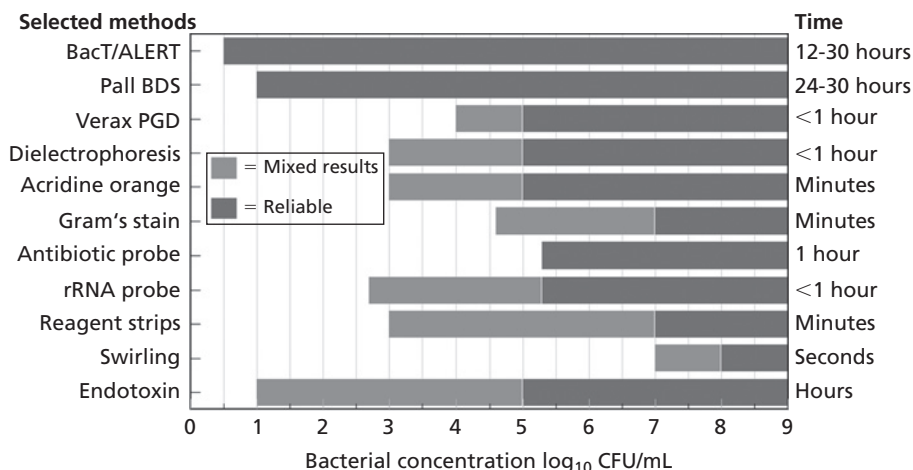


**Figure 49-3.** Methods of detection of bacterial contamination of blood components. Areas of light gray shading represent concentrations (CFU/mL) at which some, but not all, bacteria are detected by each method. Dark gray shaded areas represent ranges over which each method is reliable. The approximate time from beginning to end of the assay is reported.



### Timing of Detection

Using an automated detection system (Bactec, Becton Dickinson, Cockeysville, MD) Blajchman et al cultured WBD platelets concentrates on Days 1 (16,290 platelet concentrates) and 3 (10,065 platelet concentrates) following preparation.<sup>38</sup> Of the 16,290 platelet concentrates cultured on the day of collection, 4 units were found to be positive; however, an additional 3 units that were culture negative on the day of collection were culture positive after 2 additional days of storage. On the basis of these results the authors concluded that cultures from the day of collection may be inadequate to detect all contaminated platelet concentrates.

Bacterial growth characteristics were reported for 165 platelet units, each inoculated on the day of collection with one of the following organisms: *B. cereus*, *P. aeruginosa*, *Klebsiella pneumoniae*, *S. marcescens*, *S. aureus*, and *S. epidermidis*.<sup>72</sup> All examples of *B. cereus*, *P. aeruginosa*, *K. pneumoniae*, *S. marcescens*, and *S. aureus* had concentrations  $\geq 10^2$  CFU/mL by Day 3 following inoculation. By Day 4, all units with these organisms contained  $\geq 10^5$  CFU/mL. Units contaminated with *S. epidermidis*

showed slower and more varied growth.<sup>75</sup> This study concluded that an assay capable of detecting  $10^2$  CFU/mL on Day 3 of storage would detect a vast majority of bacterially contaminated platelet units.

In the case of red cell contamination, inoculation experiments of whole blood with *Y. enterocolitica* have demonstrated an initial rapid decline in the number of viable organisms followed by a resumption of growth after a lag phase of approximately 3 to 14 days.<sup>11,76-80</sup> During this lag phase, *Yersinia* typically cannot be recovered from samples obtained from the bag.

### Visual Inspection of Red Blood Cells to Detect Color Change

It is known that in bacterially contaminated RBC units, the attached tubing segments almost invariably remain sterile even when the organism is cultured from the blood bag.<sup>9,10</sup> An observation first noted by Kim et al and subsequently confirmed by multiple authors is a darkening of the color of unit as compared to the attached segments in additive solution RBC units.<sup>80-83</sup> Compared with sterile units, the contaminated units became noticeably darker in color 1.5 to 2 weeks after the organism was

**Figure 49-4.** Growth curves of 4 units of AS-3 RBCs inoculated with *Serratia liquefaciens* (2 units inoculated to 4.2 CFU/mL) and *Yersinia enterocolitica* (2 units inoculated to 11.6 CFU/mL). Bacteria was not detectable by culture until Days 3 to 6. Isolates were from strains actually implicated in posttransfusion sepsis (the isolates were kindly provided by M. Arduino, Centers for Disease Control and Prevention, Atlanta, GA). Used with permission from Brecher et al.<sup>74</sup>

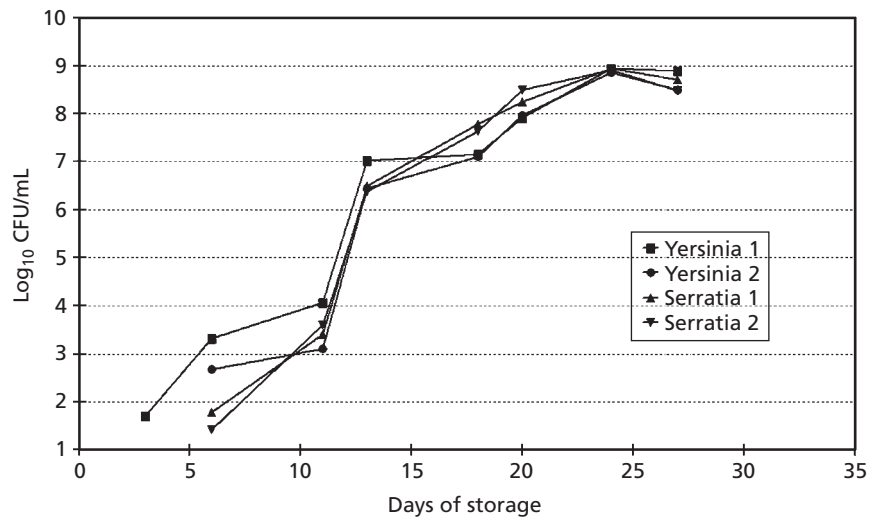
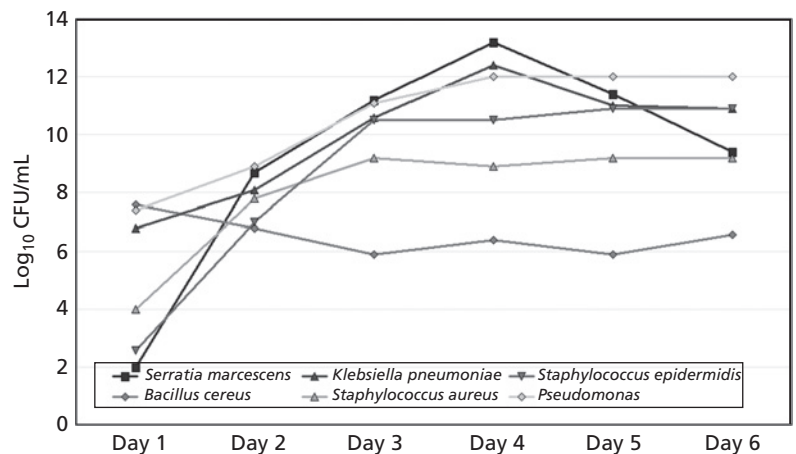


**Figure 49-5.** Growth curves of six bacteria species (*Serratia marcescens*, n = 7; *Klebsiella pneumoniae*, n = 21; *Staphylococcus epidermidis*, n = 21; *Pseudomonas sp.*, n = 15; *Bacillus cereus*, n = 9; and *Staphylococcus aureus*, n = 22) in 95 platelet units. All bacteria were inoculated on Day 0 at 10 to 50 CFU/mL. Used with permission from Brecher and Hay.<sup>7</sup>



detected in culture. This color change was very apparent when the contaminated units were compared to the attached segments of tubing, which did not darken. The sensitivity of visual identification of bacterial contamination in this manner is approximately  $10^8$  CFU/mL. One study found bacterial concentrations in the range of  $1.8 \times 10^4$  to  $1.6 \times 10^9$  CFU/mL at the time the whole blood units were first identified by visual inspection as potentially contaminated.<sup>83</sup>

#### Microscopic Examination with Gram's Stain and Acridine Orange

Microscopic examination has been used to detect bacterial contamination in both red cell components and platelets. Several authors have reported the use of the Gram's stain for microscopic examination of blood components.<sup>84,85</sup> One institution implemented a surveillance program that included pretransfusion Gram's stains of platelet pools and apheresis units.<sup>80</sup> In platelets aged 1 to 5 days, the sensitivity and specificity of this method were 80% and 96% to 99%, respectively. In 4- to 5-day-old platelets, the sensitivity increased to 100%, with a specificity of 99.3%. The true-positive results were associated with bacterial concentrations  $>10^6$  CFU/mL. Based on their analysis, these authors concluded that the use of the pretransfusion Gram's stain, combined with culture of 4- to 5-day-old units, was more cost-effective than discarding WBD platelets stored for 5 days. However, Barrett et al<sup>84</sup> reported a higher rate of false positives. In their study, eight of 5334 platelet units yielded positive Gram's stain results, six of which were negative by culture.<sup>84</sup> Other investigators have reported on the use of acridine orange as a stain for bacteria.<sup>76,86</sup>

#### Surrogate Markers

Brecher et al<sup>87</sup> described a decrease in  $PO_2$  and an increase in  $PCO_2$  (with an associated decrease in pH) in platelets that supported the growth of *S. epidermidis*. The units, which had initial  $PO_2$  levels of 79 to 137 mmHg, often showed  $PO_2$  levels below 20 mmHg by the time the organisms reached a plateau concentration of  $10^7$  to  $10^8$  CFU/mL. Other groups have explored this method.<sup>88-91</sup> Although this technique has the added advantage of being potentially noninvasive, it detects contamination only at high levels. Because platelets (and white cells) also produce carbon dioxide, this complicates attempts to detect bacterial contamination by measurements of this analyte.

Because proliferating bacteria consume glucose and generate acid, several investigators have explored the use of dipsticks to indicate metabolic changes consistent with bacterial proliferation.<sup>92,93</sup> Prospective surveillance of 3000 platelet concentrates at the M.D. Anderson Cancer Center in using reagent strips found 12 platelet concentrates with a glucose concentration or pH outside the reference range.<sup>94</sup> Two of 12 platelet concentrates were found to be culture positive for *B. cereus*. The remaining 10 platelet concentrates did not demonstrate bacterial proliferation upon culture.

Many users of WBD platelets employ the surrogate markers because culture would require a large proportion of the unit's

volume. Unfortunately, the rate of detection of the surrogate markers is much less than bacterial culture. The 2004 survey of AABB institutions showed the true-positive rate for institutions using culture was 1 in 4028 compared to a true-positive rate of 1 in 18,535 for institutions using a nonculture method.<sup>59</sup> This represents a 4.6-fold difference in the true-positive rate between culture and nonculture methods.

#### Endotoxin Assay

Investigators have used the *Limulus* amebocyte lysate assay for detection of endotoxin in blood components.<sup>11,77</sup> This method is limited to detection of endotoxin-producing organisms such as *Y. enterocolitica*.

#### DNA/RNA Techniques

Several amplification methods have been described for the detection of bacterial contamination. Schmidt et al<sup>95</sup> reported on the use of RNA polymerase chain reaction (PCR) to detect *S. aureus*, *E. coli*, *B. cereus*, and *K. pneumoniae* in pooled platelet concentrates. At an inoculum of 10 CFU/mL, the PCR testing detected all four bacteria at 12, 16, 20, and 24 hours after spiking. With a lower inoculum of 10 CFU/bag, the PCR testing detected 60% of *E. coli*, 80% of *B. cereus*, 90% of *K. pneumoniae*, and 100% of *S. aureus* 12 hours after spiking. Another study compared DNA PCR with the automated culture system BacT/ALERT.<sup>96</sup> A total of 2146 platelet concentrates were tested with both methods. When comparing to the culture method, the PCR had a sensitivity and specificity of 100%. To date, because of the complexities associated with such tests, nucleotide-based amplification has not been routinely applied to bacterial screening of platelets. One group described the use of a nonamplified chemiluminescence-linked universal bacterial rRNA probe.<sup>67,87</sup> This method uses an acridinium ester-labeled single-stranded DNA probe complementary to highly conserved bacterial rRNA regions. Although a multicenter trial confirmed the effectiveness of this technique in detecting platelet samples contaminated with one of four bacterial species, the company developing this assay chose to not pursue further assay development.

#### Rapid Immunoassay

In September 2007, the FDA approved a new rapid immunoassay for the detection of aerobic and anaerobic bacteria in leukocyte-reduced apheresis platelets as an adjunct quality control device. The Platelet PGD Test (Verax Biomedical, Worcester, MA) is a qualitative immunoassay that differentiates between gram-positive and gram-negative bacteria. As approved, it is intended to be an adjunct test after the use of an FDA-cleared bacterial culture method. The Platelet PGD Test can detect *B. cereus*, *C. perfringens*, *E. aerogenes*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *S. epidermidis*, and *S. agalactiae* at a level  $\leq 10^5$  CFU/mL and *S. marcescens* at a level of  $8.6 \times 10^5$  CFU/mL. The test takes approximately 25 minutes to perform and is optimally performed after at least 72 hours of platelet storage. The system is approved only for leukocyte-reduced apheresis platelets and has not been validated in WBD platelets.

### Bacterial Culture

There are currently two FDA-approved culture systems for bacteria detection in platelets in the United States. One uses an automated liquid culture system that includes broth bottles containing colorimetric sensors that change color as a consequence of increasing CO<sub>2</sub> produced by bacterial proliferation. The system (BacT/ALERT) monitors both the rate of change of the colorimetric sensor and the absolute color change of the sensor. The bottles are inoculated with a needle, rendering the system not completely closed. This creates the possibility of introducing bacteria into the bottle. The method reliably detects contamination of platelets inoculated to 10 CFU/mL and in many cases  $\leq 5$  CFU/mL (eg, *B. cereus*, *S. marcescens*, *C. perfringens*, *S. epidermidis*, *S. pyogenes*, *E. coli*, *K. pneumoniae*, *S. aureus*, and viridans streptococci) in 12 to 26 hours.

The ARC experience for 2004 through 2006 with the BacT/ALERT testing of 1,004,206 apheresis platelets showed a true-positive rate of 30.3% of all initially positive cultures.<sup>45</sup> A true-positive is defined as concordance between the initial culture and an additional confirmatory sample, a second 4 to 5 mL taken from the unit or its co-component. The false-positive rate was 57%, 32.4% caused by sampling contamination and 24.5%

caused by instrument signal error. The remainder of the initially positive cultures (12.7%) was called indeterminate, defined as the component not being available for confirmatory testing. In total, 612 donations had an initial positive culture result. Ninety-seven platelet units had been transfused at the time of the positive results; and only one of the 97 was confirmed positive, for coagulase-negative *Staphylococcus*. This unit was transfused on Day 2 and the patient did not have signs of sepsis. No septic reactions were noted with the units associated with an indeterminate or the false-positive culture.

Blood Systems, another large collection system in the United States, also reported their first 2 years of experience with culturing apheresis platelets and WBD platelets.<sup>97</sup> With the apheresis platelets, 198 of 122,971 collections initially tested positive and 21 were confirmed positive to give a confirmed positive rate of 1 in 5568. No confirmed positives were transfused. A total of 50 false-positive results were seen, caused by either machine reading error or contamination, and 26 cases were indeterminate. For WBD platelets, a total of 13,579 were cultured with 12 initial positives, one true positive, and 11 false positives. Data from ARC and Blood Systems are summarized in Table 49-9 and Fig 49-6.

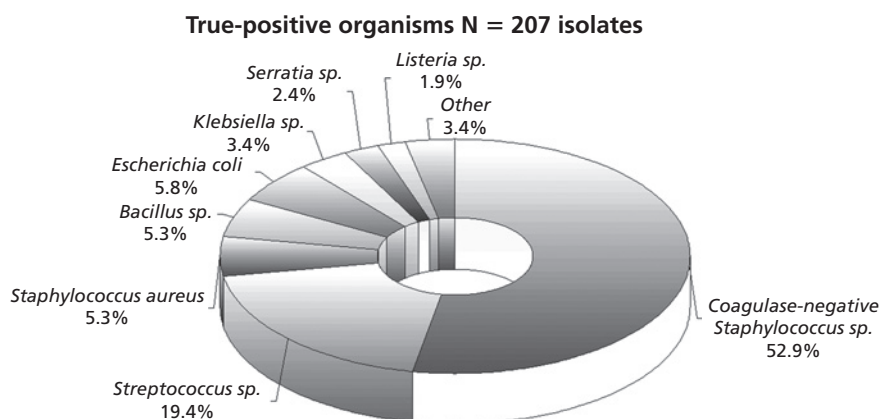
**Table 49-9.** Summary of Experience with Bacterial Culture at ARC and Blood Systems\*

|                                     | True Positive (%)      | False Positive (%) | Indeterminate (%) |
|-------------------------------------|------------------------|--------------------|-------------------|
| <b>Gram positive</b>                | 77 (86.5)              | 200 (98.5)         | 59 (92.2)         |
| <i>Staphylococcus</i> sp.           | 43 (48.3)              | 53 (26.1)          | 15 (23.4)         |
| <i>Bacillus</i> sp.                 | 7 (7.9)                | 117 (57.6)         | 28 (43.8)         |
| <i>Streptococcus</i> sp.            | 21 (23.6)              | 5 (2.5)            | 3 (4.7)           |
| <i>Diphtheroids/Corynebacterium</i> | 1 (1.1)                | 10 (4.9)           | 8 (12.5)          |
| Other                               | 5 (5.6)                | 15 (7.4)           | 5 (7.8)           |
| <b>Gram negative</b>                | 12 <sup>†</sup> (13.4) | 2 (1.0)            | 2 (3.1)           |
| Fungal                              | 0 (0)                  | 1 (0.5)            | 3 (4.7)           |
| <b>Total</b>                        | 89 (100)               | 203 (100)          | 64 (100)          |

\*Used with permission from Pietersz et al.<sup>98</sup>

<sup>†</sup>True-positive gram negatives included *Serratia marcescens* (n = 4), *Klebsiella* sp. (n = 3), *Escherichia coli* (n = 3), *Citrobacter diversus* (n = 1) and unspecified gram-negative rod (n = 1).

**Figure 49-6.** True-positive (confirmed) organisms, isolated from 1,237,177 apheresis cultures (tested with 4 mL in an aerobic BacT/ALERT bottle). Examples of organisms isolated only once or twice were grouped in the "other" category. These included example(s) of *Micrococcus* sp., *Diphtheroids/Corynebacterium*, *Enterococcus avium* (n = 2), *Granulicatella adiacens*, *Citrobacter* sp., *Lactobacillus* sp., and *Enterobacter aerogenes*. Used with permission from Pietersz et al.<sup>98</sup>



The second culture system is the enhanced Pall Bacterial Detection System (eBDS). The eBDS system measures the oxygen in the headspace gas of the sample pouch. Because the system measures oxygen consumption, it will not detect obligate anaerobes. One study reported on the use of the eBDS system with 118,067 apheresis and WBD units from 23 US blood centers.<sup>98,99</sup> Investigators found 118 initially reactive (1 in 992) and 23 confirmed positive (1 in 5133) units. All true-positive units contained *Staphylococcus* spp. and *Streptococcus* spp. Ninety-five false-positive units were identified (1 in 1243). Of the false positives, 18 were caused contamination and 56 had no bacteria present. There was one report of a missed detection with *S. epidermidis*. In a study by Fourneir-Wirth et al, 5 to 50 CFU/mL bacteria was inoculated into apheresis platelet units and tested in the eBDS.<sup>100</sup> No false-positive results were seen. After 18 hours of incubation, 61 of 63 units tested positive and after 24 hours, all 63 tested positive. In the earlier incubation, two cases of *B. cereus* were missed.

### Sampling and Bottle Types for Culture

The BacT/ALERT system can be used either with one aerobic bottle or with one aerobic and one anaerobic bottle. In 2004, approximately half of hospital blood banks used only the aerobic bottle, which is inoculated with 4 mL of the platelet product.<sup>59</sup> Although most bacteria involved in septic transfusion reactions are aerobic, certain aerobic bacteria grow faster in the anaerobic bottle, most likely because of the difference in the nutrient broth.<sup>101-107</sup> One study used a strain of *Staphylococcus lugdunensis* to perform a variety of spiking experiments to investigate the growth characteristics in the two types of bottles.<sup>108</sup> The strain of *S. lugdunensis* was implicated in a fatal septic reaction to a platelet unit. Using a 4-mL inoculum, the anaerobic bottle became positive at a mean time of 18.73 hours as compared to the aerobic bottle at 21.07 ( $p < 0.001$ ). As the number of organisms inoculated into each bottle was increased, the difference in time to reactivity decreased (Fig 49-7). Because most centers use 4 mL per bottle, the use of an anaerobic bottle could reduce the time to detection and possibly prevent the transfusion of contaminated units.

In 2005, the FDA approved the Post Approval Surveillance Study of Platelet Outcomes, Release Tested (PASSPORT) for use

with apheresis platelets collected with caridianBCT (Lakewood, CO) and Baxter (Deerfield, IL) equipment. This study allows centers to extend the expiration of these apheresis platelets to 7 days if the units are cultured with both an aerobic and an anaerobic bottle. The University of North Carolina recently reported its experiences with regard to inventory impact as a result of the extended shelf life.<sup>109</sup> In total from March through August 2006, of the 1688 platelets transfused, 139 were older than 5 days. Since the implementation of 7-day platelets, the mean day of issue was 4.02 as compared to 3.44 before the extended shelf life. Additionally, the percent of outdating decreased from 2.8% to 1.3% with the extended shelf life. In the study period, 6 units were removed from inventory for an initial positive culture, none of which was confirmed. Only one allergic reaction was noted to a 7-day platelet, but no other adverse events were reported. Due to the higher than expected culture positive rate with outdated platelets, the passport study has been suspended.

### Causes of False-Negative Results

Even though great strides have been made in the prevention of platelet septic transfusion reactions, there are still missed cases. Missampling, micropunctures, leakage, postsampling handling, and contaminated tubes or container surfaces are all possible causes of contamination.<sup>110</sup> WBD platelets have presented a problem because of the difficulty with culture, but with the introduction of the Acrodose prestorage pooling system, more contaminated WBD platelets can be interdicted before transfusion. With the recent FDA clearance of the Verax system, contaminated units that are culture negative might be interdicted immediately before transfusion (although this would be an off-label use if used with WBD platelets). Reduced morbidity and mortality of platelet transfusion is also achieved with the optimal and correct use of blood components.<sup>47</sup>

### Bacterial Elimination

#### Filtration

Leukocyte reduction of blood components is widely used in North American, Europe, Asia, and Australia. Several countries,

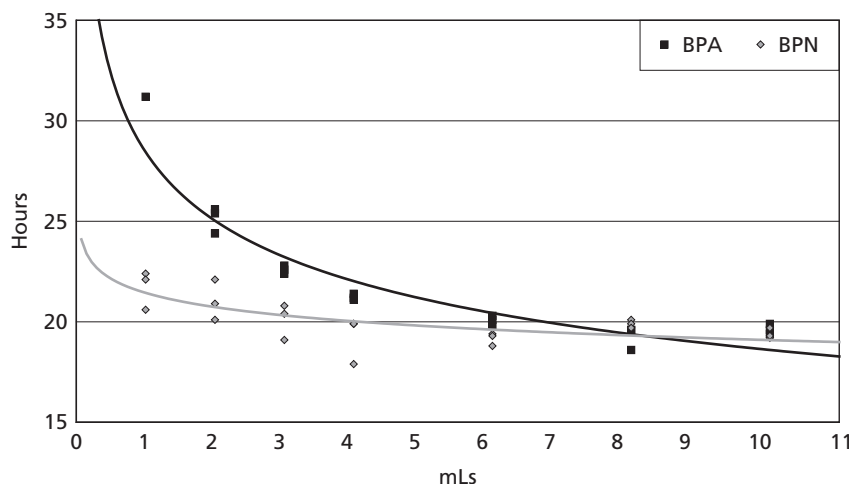


**Figure 49-7.** Time to reactivity of the aerobic (BPA) and the anaerobic (BPN) bottles inoculated at 1.5 CFU/mL with increasing volume. At lower inocula, the reactive BPN bottles are faster than the BPA bottles; at higher inocula, the time to reactivity were equivalent. Used with permission from Brecher and Hay.<sup>108</sup>

including Canada, England, Portugal, France, Ireland, and Norway, have switched to a completely leukocyte-reduced blood supply (so-called “universal leukocyte reduction”).<sup>111,112</sup>

Because of concern regarding the accumulation of cytokines, leukocyte and platelet breakdown products, and possibly immunogenic white cell fragments in stored blood, emphasis has been placed on prestorage rather than poststorage leukocyte reduction.<sup>113-115</sup> A theoretic risk of prestorage filtration is that early removal of the phagocytic leukocytes, which would normally remove low levels of bacteria present in these products, might lead to an increase in bacterial contamination of blood components and septic complications. In the case of *Y. enterocolitica* (the organism that has been most extensively studied), prestorage leukocyte reduction by filtration actually is associated with a decrease in bacterial growth in inoculated RBCs.<sup>4,76,77,116-118</sup> (Table 49-10).

The mechanism by which leukocyte reduction removes bacteria is multifactorial. Bacteria that have been phagocytosed but not killed are removed with the white cells. Alternatively, organisms may be adsorbed to leukocytes or activated by complement, to then be bound indirectly to the charged filter fibers, or the bacteria may directly adhere to the filter fibers. AuBuchon and Pickard showed with a panel of bacterial organisms, differences in the affinity for bacteria by direct adherence of red cell leukocyte reduction filters and platelet leukocyte reduction filters.<sup>119</sup> In general, red cell reduction filters are most efficient, removing 88% to 100% of organisms, while platelet filters remove only 67% to 97% of organisms. Unlike the decrease in *Y. enterocolitica* growth seen with filtered, prestorage leukocyte-reduced red cells, leukocyte reduction by filtration failed to decrease bacterial growth in platelets spiked with bacteria.<sup>120-121</sup>

### Prolonged Room Temperature Hold of Red Blood Cells

When whole blood is placed immediately at 4°C, bacterial growth occurs more rapidly than if held at 10°C for 24 hours before 4°C storage.<sup>123</sup> Bacterial growth of most pathogens is inhibited by cold temperatures, but host mechanisms present in fresh whole

blood are also inhibited at 4°C. With plasmid-encoded complement resistance, *Yersinia* can be complement sensitive when blood is transiently stored at 20°C.<sup>73</sup> Because phagocytosis and complement activation are impaired at 4°C, it may be advantageous to allow red cells to remain in contact with plasma for several hours before separation and storage of components.

### Pathogen Inactivation

Several techniques to inactivate pathogens in blood components using chemicals are under active investigation or are implemented in select countries (see Chapter 51).

### International Comparison

There is wide variation in the way different countries approach the problem of bacterial contamination of platelets. A recent survey of 12 different countries reveals the many approaches to preventing septic transfusion reactions.<sup>98</sup> The responses are summarized in Table 49-11.

## Transfusion-Transmitted Syphilis

*Treponema pallidum* is a thin-walled, motile, spiral gram-negative rod or spirochete that cannot be visualized with Gram's stain and does not grow on bacteriologic media or cell culture. Although it is a bacterium, it is often treated as a distinct entity, different from other transfusion-transmitted bacterial organisms and thus is addressed separately. Only 25% of patients with primary syphilis have a reactive serologic test for syphilis and the test does not become routinely positive until the fourth week after the onset of symptoms; therefore, donors infected with *T. pallidum* may be asymptomatic with negative serology during periods of spirochetemia.<sup>124,125</sup> Although the organism does not survive prolonged storage at 4°C, it may live for 1 to 5 days at these cold temperatures.<sup>126,127</sup> Therefore, a rare infection may be associated with transfusion of a fresh RBC unit from a donor who was in the seronegative phase at the time of

**Table 49-10.** Selected Studies of *Yersinia enterocolitica* Growth and Prestorage Leukocyte Reduction by Filtration

| Inoculating Concentration CFU/mL | Filtered Growth/Total (%) | Control Growth/Total (%) | Filter Type                             | p Value* | Reference                    |
|----------------------------------|---------------------------|--------------------------|-----------------------------------------|----------|------------------------------|
| 100                              | 0/10 (0)                  | 10/10 (100)              | Sepacell PL-5N                          | <0.001   | Brecher et al <sup>104</sup> |
| 65                               | 2/10 (20)                 | 8/8 (100)                | Leukotrap and Leukotrap RC (Pall RC300) | 0.002    | Brecher et al <sup>72</sup>  |
| 10/150                           | 3/8 (37)                  | 8/8 (100)                | Pall BPF4                               | 0.03     | Brecher et al <sup>102</sup> |
| 0.3-132                          | 3/24 (12)                 | 16/24 (67)               | Sepacell R-500                          | <0.001   | Hoppe <sup>73</sup>          |
| 20-30,000                        | 6/30 (20)                 | 22/30 (70)               | Cellselect, NPBI                        | <0.001   | Brecher et al <sup>105</sup> |
| 1.5                              | 2/6 (33)                  | 6/6 (100)                | Leukotrap RC (Pall RC300)               | 0.06     | Brecher et al <sup>103</sup> |
| Total                            | 16/88 (18)                | 70/86 (81)               |                                         | <0.001   |                              |

\* Two-sided Fisher's exact test.

**Table 49-11.** Comparison of Methods Used in Different Countries to Address the Bacterial Contamination of Platelets<sup>98</sup>

| Country        | Routine Screening for Bacterial Contamination                                                                                                                | Method Used for Screening                                                                                                                                                                                                                                | Impact of Testing on Logistics and Outdating                                                                                                  |
|----------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------|
| Australia      | 5% of platelet concentrates (PCs) are tested; 100% tested beginning in April 2008                                                                            | Bact/ALERT; cultures taken 24 hours after collection for apheresis platelets and 48 hours after for pooled platelets; both aerobic and anaerobic bottles                                                                                                 | Predicted that outdating will increase 1-5% with 100% testing, using a 24-hour hold before sampling                                           |
| Austria        | Approximately 5% of apheresis platelets are tested for quality control (QC)                                                                                  | Bact/ALERT; both aerobic and anaerobic bottles                                                                                                                                                                                                           | No effect because testing duration is 7 days and platelet shelf life is 5 days                                                                |
| Canada         | Approximately 98% of apheresis platelets and 4-unit buffy coat (BC) platelet pools are tested; once all whole-blood derived units are BC, all will be tested | Bact/ALERT; aerobic bottle only; tested 24-30 hours after collection                                                                                                                                                                                     | No effect because no quarantine after the culture sample is drawn                                                                             |
| Denmark        | Currently, 2/3 of PCs are tested; the National Board of Health will hopefully recommend that all be tested in the near future                                | Bact/ALERT or corresponding culture methods; sampled 3-30 hours after collection of whole blood (0-24 hours after pooling of BC or collection of apheresis units)                                                                                        | Shelf life increased from 5 to 7 days with culture; outdating decreased from 15% to 5%                                                        |
| France*        | None                                                                                                                                                         | Not applicable                                                                                                                                                                                                                                           | Not applicable                                                                                                                                |
| Germany        | Done for QC only                                                                                                                                             | Bact/ALERT with both aerobic and anaerobic bottles                                                                                                                                                                                                       | Not applicable                                                                                                                                |
| Ireland        | On all platelets since 2004                                                                                                                                  | Bact/ALERT; for platelet pools—platelets incubated 36 hours before culture; for apheresis platelets—incubated 12 hours before culture; use both aerobic and anaerobic                                                                                    | Modest improvement in logistics because of retesting at Day 4 of platelets collected on caridianBCT Trima and then shelf life extended 2 days |
| Japan          | No, platelet shelf life only 72 hours                                                                                                                        | Not applicable                                                                                                                                                                                                                                           | Not applicable                                                                                                                                |
| Netherlands    | On all platelets since 2001                                                                                                                                  | Bact/ALERT; both aerobic and anaerobic bottles; sampled at preparation and issued as “negative to date” as soon as culture begins                                                                                                                        | Outdating decreased because of extension of shelf life from 5 to 7 days                                                                       |
| Norway         | On all platelets at major hospitals since 1998                                                                                                               | Bact/ALERT using only aerobic bottles sampled the day after culture of the starting material (either whole-blood-derived or apheresis platelets); no quarantine after culture                                                                            | Outdating decreased from 18% to 11% based on extending shelf life to 6.5 days                                                                 |
| United Kingdom | In England, only for QC or tested in order to extend the shelf life over holidays; in Scotland, Wales, and in Northern Ireland all PCs are routinely tested  | If used to extend shelf life: sampled on Day 3 into a Bact/ALERT aerobic bottle and quarantined for 48 hours<br><br>If used in retrospective national monitoring study: Bact/ALERT aerobic and anaerobic bottles are inoculated and incubated for 7 days | Extending the shelf life has assisted with supply over extended holidays                                                                      |
| United States  | No governmental mandate but required by AABB and CAP                                                                                                         | Two systems FDA approved and available for testing: Bact/ALERT, eBDS, all units sampled after 24-36 hour hold; whole blood derived platelets tested with surrogate markers such as glucose or pH                                                         | No or modest impact on availability as well as no increase in outdating                                                                       |

\* Pathogen inactivation is being used on Reunion Island because of the risk of chikungunya transmission; being studied in other French locations and will be implemented gradually.

donation. Platelets stored at 20 to 24°C, provide a more hospitable temperature for *T. pallidum*; however, this organism does not thrive with the high oxygen tension in modern platelet storage bags. Since 1969, only three cases of transfusion-transmitted syphilis have been reported in the literature.<sup>128-130</sup> The extremely low rate of transfusion-transmitted syphilis infection likely results from 1) donor questioning targeting high-risk behavior; 2) the cardiolipin-based assay, although an insensitive test in the acute postinfectious setting, does pick up a number of infected donors; 3) refrigerator storage, which results in the death of spirochetes; 4) antibiotics given to many patients at the time of platelet transfusion, which would be bactericidal for any viable organisms; and 5) donors excluded for a positive test for HIV, HCV, or hepatitis B because of the high correlation between infection with *T. pallidum* and viruses such as these, even though the donors may have been in the seronegative phase of syphilis at the time of donation.<sup>131-136</sup> Because syphilis testing plays only a minor role in protecting the blood supply and is associated with a high degree of false-positive reactions, elimination of syphilis testing has been advocated. The counter argument is that such testing provides a surrogate marker for individuals at risk of other sexually transmitted diseases and, therefore, should be retained.

## Transfusion-Transmitted Lyme Disease

Lyme disease, a tick-borne illness, caused by the spirochete *Borrelia burgdorferi* is theoretically transmittable through blood, although no reported human transfusion-transmitted cases have been identified.<sup>137</sup> The tick that carries *B. burgdorferi*, *Ixodes scapularis*, also carries *Babesia microti* and *Anaplasma phagocytophilum*, both of which are transmissible by transfusion. In a murine model of Lyme disease, approximately 50% of mice transfused blood from a spirochetemic donor mouse became infected.<sup>138</sup> Although there have been cases of blood donors being diagnosed with Lyme disease shortly after their donation, no spirochetes could be identified in the recipient of the units.<sup>139</sup> This might be unrecognized because of vague symptoms of Lyme disease as well as the lack of characteristic erythema migrans at the site of tick bite.<sup>137,140</sup> Additionally, spirochetes are rarely identified even in patients with active disease, which may in turn limit the transmission through transfusion.<sup>139</sup>

## Conclusion

Bacterial contamination of blood components is the most common cause of transfusion-transmitted infectious disease. Most cases of posttransfusion sepsis involve platelets that must be stored at room temperature. With the introduction of diversion techniques and bacteria detection of platelets, the rate of clinically significant septic reaction has substantially decreased and the availability of platelets to patients has been enhanced.

## Disclaimer

Y. Park has disclosed no conflicts of interest. M. Brecher has disclosed relationships with bioMérieux, Pall, Fenwal, Verax and Cerus.

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Prion diseases, or transmissible spongiform encephalopathies (TSEs), comprise a spectrum of diseases in animals and humans. In animals, these diseases include scrapie in sheep and goats, chronic wasting disease in deer and elk, and transmissible mink encephalopathy. Bovine spongiform encephalopathy (BSE) was first described in cattle in the United Kingdom (UK) in the early 1980s and developed into an epidemic of more than 180,000 bovine cases, spreading also to a range of other animals including domestic and exotic cats and exotic ungulates.

Sporadic or classical Creutzfeldt-Jakob disease (CJD), first described in the early 1920s,<sup>1</sup> occurs at an incidence of around 1 in 1 million per year. It presents at a mean age of 68 years with a rapidly progressive dementia, leading to death after about 6 months. Kuru, described in the Fore people of Papua New Guinea,<sup>2</sup> comprised an endemic disease presenting with cerebellar ataxia and a more prolonged clinical course. The disease is thought to have been spread through cannibalistic funeral rites. Although these cultural practices died out toward the end of the 1950s, there are still occasional people who develop new clinical disease, pointing to the potentially very prolonged incubation periods of these diseases. Iatrogenic transmission of sporadic CJD has occurred via neurosurgical instrumentation and electroencephalogram (EEG) electrodes, corneal and dura mater grafts, and cadaveric pituitary-derived growth and follicular-stimulating hormones.<sup>3</sup> Those patients infected through direct central nervous system inoculation tend to manifest an incubation period of around 2 years and a rapidly progressive dementia reminiscent of sporadic CJD. Those infected through peripheral inoculation demonstrate a more prolonged and variable incubation period (mean 13–15 years) and a clinical syndrome similar to that of Kuru, suggesting that the route of infection has a significant impact on the incubation period and clinical manifestation of disease.

Finally, there are a group of rare inherited human prion diseases—including familial CJD, Gerstmann-Sträusler-Scheinker (GSS) disease, and fatal familial insomnia—related to polymorphisms within the gene for prion protein.<sup>4</sup> The pathology of these disorders is characterized by the accumulation of abnormal prion protein (PrP<sup>TSE</sup>) within the central nervous system associated with neuronal degeneration and reactive gliosis, giving rise to the characteristic spongiform appearance from which these diseases took their original name.<sup>5</sup> There is patchy evidence of peripheral accumulation of PrP<sup>TSE</sup> in patients with sporadic and familial forms of CJD, but little substantive evidence of infectivity in peripheral blood and tissues or of transmission by blood components, plasma derivatives, or cellular, tissue, or organ transplants.<sup>6</sup>

Variant CJD was first described in 1996.<sup>7</sup> The disease is characterized by a relatively early onset compared to sporadic disease (median 28 years, range 12–74 years), a clinical presentation consisting of behavioral disturbance,<sup>8</sup> dysesthesia and ataxia followed by progressive neurologic deterioration,<sup>9</sup> and a prolonged clinical phase (median 14 months, range 6 to 48 months).<sup>10</sup> The pathologic features are also characteristic, with the presence of PrP<sup>TSE</sup> not only within the central nervous system, but also in the follicular dendritic cells of peripheral lymphoid tissues including tonsils, spleen, lymph nodes, and gut-associated lymphoid tissue. PrP<sup>TSE</sup> has been demonstrated in two appendix samples removed 8 months and 2 years before the onset of clinical disease.<sup>11</sup> The epidemiologic, pathologic, and experimental data are consistent with variant CJD having arisen from the oral transmission of BSE from infected cattle.

To date, 167 cases have been described in the UK, 23 in France, four in Ireland, three each in the US and Spain, two each in the Netherlands and Portugal, and one each in Canada, Japan, Saudi Arabia, and Italy.<sup>12</sup> Two of the Irish patients, two of those in the US, and those in Canada and Japan are thought to have been infected during travel in the UK. The overall incidence of clinical cases appears to be diminishing, with current mathematical models projecting a maximum likelihood of 70 [95% confidence interval (CI), 10–190] further clinical

cases in the UK.<sup>13</sup> However, the possibility of a cohort of subclinically infected individuals<sup>14</sup> and four cases of transmission of variant CJD prions by blood components described in 2004 and 2006<sup>15-17</sup> raise concern that secondary and higher order transmissions by blood components, plasma derivatives, and cellular, tissue, or organ transplantation could extend the outbreak, particularly if compounded by other potential routes of transmission such as surgery and interventional medical procedures. Although blood service organizations have taken several precautionary measures,<sup>18,19</sup> the persisting uncertainties surrounding the nature, level, and distribution of infectivity; the prevalence of subclinical disease; and the overall transmissibility of the disease undermine any ability to accurately judge the magnitude of this risk and evaluate the impact of current and proposed risk management strategies.

## The Molecular Basis of Prion Diseases

Prion diseases are associated with a change in the secondary and tertiary conformation of a widely expressed glycoprotein termed prion protein (PrP<sup>C</sup>). PrP<sup>C</sup> is a 30- to 35-kD protein with two N-linked glycosylation sites and a glycosylphosphatidylinositol anchor. The secondary structure consists of around 40% alpha-helix and 3% to 4% beta-pleated sheet. Prion infection is associated with a conformational transformation, resulting in an increase in the proportion of beta-pleated sheet. This, in turn, engenders a change in the physicochemical and biological properties of the molecule (PrP<sup>TSE</sup>), including an increased resistance to degradation of biological and physical agents (PrP<sup>RES</sup>).<sup>20</sup> The precise etiology of the conformational change remains unclear, with some authorities favoring a process of homodimerization and others preferring one of nuclear polymerization. The prion hypothesis<sup>21,22</sup> proposes that PrP<sup>TSE</sup> is the direct cause of infectivity, although it is acknowledged that the relationship between PrP<sup>TSE</sup>, PrP<sup>RES</sup>, infectivity, and tissue damage is not straightforward. Some authorities suggest that a small nucleic acid-based agent may be the causative agent.<sup>23,24</sup>

## The Nature, Concentration, and Distribution of Infectivity

The current understanding of the pathogenesis of CJD is largely predicated on animal studies. Biochemical and biological assays point to the accumulation of high concentrations of PrP<sup>TSE</sup> and infectivity in the central nervous system in all forms of CJD. Animal models of peripherally transmitted prion diseases,<sup>25,26</sup> point to follicular dendritic cells (FDCs)<sup>27,28</sup> in the germinal centers of lymphoid tissue as the key cell in the establishment of infection and not B lymphocytes as previously suggested.<sup>29,30</sup> The presence of abnormal prion accumulation in the FDCs in tonsil, spleen, and lymph nodes of all patients with variant CJD thus far examined postmortem suggests a similar pathophysiology

for CJD in humans following primary (eg, cattle BSE to humans) or secondary and higher order (human to human) transmission.<sup>31</sup>

The exact nature of infectivity in peripheral blood and tissues is uncertain. Recent work suggests that in brain homogenates, the maximum specific infectivity is associated with proteinase-resistant particles of 300 to 600 kD (ie, oligomers of 14 to 28 PrP molecules).<sup>32</sup> There are also data suggesting that proteinase sensitive forms of PrP<sup>TSE</sup> could be infectious. Further work in this area, particularly relating to the physicochemical characteristics of the infectious prion in plasma is a priority.

The World Health Organization (WHO) has reviewed the data on the distribution of infectivity and PrP<sup>TSE</sup> in peripheral tissues and organs in human prion diseases and in naturally and experimentally infected animal prion diseases.<sup>6</sup> Although the highest levels of infectivity are confined to neurologic tissues, lower levels of infectivity and/or PrP<sup>TSE</sup> have been demonstrated in a wide range of other tissues.

Two sets of rodent studies are informative with regard to the likely concentration and distribution of infectivity in peripheral blood. The first studies were carried out in mice infected with the Fukuoka-1 strain of GSS disease<sup>33,34</sup> and provided the data on which the original risk assessments were based.<sup>35</sup> The second set of studies was carried out in hamsters infected with the 263K strain of scrapie and has led to a review of blood infectivity assumptions.<sup>36,37</sup> Overall, the current working assumption is that the level of infectivity in the peripheral blood during the incubation period of a peripherally transmitted prion disease such as variant CJD is likely to be in the order of magnitude of 10 infectious doses/mL. In humans the data on peripheral blood infectivity in sporadic and familial CJD is open to interpretation<sup>38</sup> and it has not yet proven possible to detect infectivity in the peripheral blood of patients with variant CJD, despite the fact that the disease is clearly clinically transmissible. Moreover, it is known that the distribution of PrP<sup>C</sup> varies significantly between rodents and humans.<sup>39-41</sup> There are, therefore, important caveats regarding the extrapolation of these estimates to humans and it is probably safer to consider a plausible range of infectivity in the peripheral blood of humans for the purposes of risk assessment.

The spatial distribution of infectivity also varies between models. Brown et al<sup>33,34</sup> mimicked to a certain extent clinical blood separation processes and demonstrated a four- to five-fold higher concentration of infectivity in the buffy coat (per unit volume) compared to plasma. Gregori et al studied purified blood components and concluded that red cells and platelets have very little infectivity, and that approximately 40% of infectivity is associated with leukocytes, with most of the remainder residing within the plasma.<sup>36,42</sup>

The temporal development of infectivity in peripheral tissues during the incubation period is similarly uncertain. In rodent models, a variety of patterns of change in infectivity and PrP<sup>TSE</sup> concentration have been observed.<sup>43,44</sup> In humans, the pattern of development of infectivity in peripheral blood and tissues during CJD infection is unknown, although, as noted previously,

abnormal PrP has been detected in an appendix sample removed 2 years before development of clinical variant CJD.

### Transmissibility of CJD by Blood and Tissues

In general, one would expect that transmission of infection within a species would be more efficient than that between different species.<sup>45</sup> The transmission of infection by blood drawn from a donor during both the incubation period and clinical phase of disease has been demonstrated in sheep naturally infected with scrapie and experimentally infected with BSE with an efficiency of approximately 50%, suggesting that the level of infectivity might be lower than that seen in the rodent models.<sup>46-48</sup>

Although there are a handful of reports of patients who have developed sporadic CJD following exposure to blood components,<sup>49</sup> plasma derivatives,<sup>50-53</sup> or organ transplantation,<sup>50</sup> these reports have not established a convincing link with sporadic CJD in the donors. To the contrary, a large number of epidemiologic case control,<sup>54-64</sup> look-back,<sup>65,66</sup> and surveillance<sup>67-70</sup> studies over the past 25 years have demonstrated no clear evidence of transmission of sporadic CJD by blood transfusion or plasma derivatives. It should be borne in mind, however, that CJD is a rare disease and occasional cases of transmission by blood components could have been missed.

The Transfusion Medicine Epidemiology Review was established in the UK in 1996 in order to monitor potential linkage between CJD in donors and recipients.<sup>71,72</sup> Individuals who develop variant CJD are reported to the UK Blood Services by the National CJD Surveillance Unit (NCJD-SU) to establish whether they have previously been blood donors. If they have been donors, the recipients are traced and notified. In the reverse arm, NCJD-SU determines whether individuals who have developed variant CJD have themselves received blood. If they have been transfusion recipients, the UK Blood Services trace and notify the donors. Individuals identified in this way are considered to be “at risk for public health purposes.”<sup>73</sup> There are clear ethical and social tensions in adopting a notification strategy when the level of risk is uncertain, psychologic and social detriment may ensue, and no practical benefit accrues to the notified individuals.<sup>74,75</sup> No linkage has been identified between donors and recipients with sporadic or familial CJD in this study. However, 18 UK blood donors have gone on to develop variant CJD and of their 66 traceable recipients (26 of whom are informative in the sense of having survived more than 5 years after transfusion), three have developed variant CJD and one showed evidence of subclinical infection at postmortem examination (having died of an unrelated condition).<sup>15-17</sup> In France, three blood donors have developed variant CJD, although of their 18 traceable recipients, none have developed variant CJD at the time of this writing.

Eleven of the above-noted UK blood donors also contributed 25 plasma donations to pools from which a total of 178 plasma batches were manufactured before the UK began importing plasma in 1999. No recipients of implicated products have thus

far developed clinical variant CJD—although again, following risk assessment, many of these people are now regarded as “potentially at risk for public health purposes.”<sup>73,76</sup>

Cells, tissues, and organs are much less frequently transplanted and many are derived from brain-stem dead or non-heart-beating donors. In the absence of routine cadaveric testing it is, therefore, not possible to know whether such individuals may have been incubating CJD at the time of their deaths.<sup>77</sup> There are no clearly substantiated reports of individuals having developed CJD as a result of cellular, tissue, or organ transplantation. However, both prion diseases and these transplant procedures are relatively uncommon events and given that a significant tissue mass is transplanted, the concentration of infectivity that would be required to transmit infection is well below the level of sensitivity of current assay methods. Therefore, a precautionary assumption is that these tissues may be capable of transmitting disease should the donor be infected.

### The Prevalence and Distribution of Subclinical Disease

It is the prevalence and epidemiologic distribution of pre- or subclinical disease that drives the risk of secondary transmission, rather than the incidence of clinical disease. Although the incidence of sporadic CJD is known, the incubation period is uncertain and the prevalence of pre- or subclinical disease is unknown. Iatrogenic and familial forms of CJD are fortunately rare; however, it appears likely that preclinical cases will occur in these forms of disease. Those individuals in at-risk groups can often be identified by family or medical history and excluded as donors.

The prevalence and distribution of subclinical variant CJD is also unknown; however, the available data provide some important hints.

First, the median age of onset of disease has not altered over the past decade in the way one would expect where a cohort of individuals has been exposed to the risk of infection over a discrete period.<sup>7,12,78</sup> These data suggest the existence of age-related susceptibility and/or exposure between the ages of 10 and 20 years,<sup>13,79,80</sup> a cohort who would now be between 20 and 40 years old. The patients who have developed variant CJD in France have an older age profile (median age 37 years) than in those in the UK: the reason for this difference is unknown.

The data generated by retrospective study of tonsil and appendix samples has also been revealing.<sup>14,81,82</sup> Three out of 12,674 samples were found to be positive for abnormal prion protein on Western blot,<sup>83</sup> where the specificity is thought to be high,<sup>84</sup> but the sensitivity uncertain.<sup>85</sup> Mathematical modeling suggests a maximum likelihood of 3000 infected people (95% CI, 520-6810) in the UK, mainly in the 10- to 30-year age group.<sup>13</sup> This is at variance with the number of individuals projected to develop clinical disease and suggests a probability of subclinical infection of 0.93 (95% CI, 0.70-0.97).<sup>13,86</sup> The potential existence of a cohort of individuals in the population with long-term

subclinical infection<sup>87,88</sup> is consistent with experimental animal data<sup>89,90</sup> suggesting that methionine/valine heterozygosity or valine homozygosity at codon 129 of the *PRNP* gene predisposes to a prolonged period of subclinical disease. Although all clinical cases of variant CJD thus far have been methionine homozygous at codon 129,<sup>91</sup> the second reported case of transfusion-transmitted variant CJD prions was shown to be methionine/valine heterozygous at codon 129.<sup>16</sup> Two of the three patients who tested positive in the retrospective study of tonsils and appendices have also recently been shown to be valine homozygous at this locus,<sup>92</sup> suggesting that codon 129 genotype (among other things) may influence the propensity of an infected individual to develop clinical disease. Long-term studies on patients who developed Kuru or iatrogenic CJD support this possibility. Individuals who were homozygous for methionine at codon 129 developed clinical disease sooner than those of other codon 129 genotypes, who have developed disease more sporadically and with longer incubation periods (in some cases, over 40 years from their likely infection).<sup>93</sup>

Taken together, these data suggest that the underlying prevalence of subclinical disease in the UK could be around 1 in 10,000 (range 1 in 1000 to 1 in 20,000) and that there could be further waves of clinical disease in individuals with other codon 129 genotypes. There are no specific data on which to estimate the prevalence in other countries. However, from the relative incidence of clinical disease, one might expect prevalence in Ireland to be approximately 20% of that in the UK, that in France to be approximately 10%, and that in the Netherlands to be around 5%.

## Approaches to Risk Management

In the face of the uncertainties and extrapolated estimates, a precautionary set of assumptions is that it is likely that there exists a proportion of healthy people with subclinical variant CJD in the UK and other Western European countries, that infectivity is present in the peripheral blood of these individuals, and that transmission of disease is possible through blood and tissue products, but the exact magnitude of these risks is unclear. The best risk management approaches are those that are likely to have some beneficial impact over the widest range of plausible scenarios. However, the effectiveness of such precautionary strategies may be partial or unknown and the potential for risk substitution and ethical dilemma needs to be considered.

### Donor Selection

Residence in the UK (and, to a lesser extent, Ireland and France) is clearly a risk factor for variant CJD and many countries exclude prospective donors who have lived in such countries.<sup>94</sup> The stipulated period of residence varies widely, depending on the negative impact on the blood donor base and the perceived differential risk.<sup>95</sup>

It has proved more difficult to delineate subpopulations who may be considered to be at higher risk of subclinical vCJD than the general population. Individuals considered to be “presumed

infected” or “at risk for public health purposes” are deferred from blood transfusion and tissue transplantation.<sup>73</sup> Although blood transfusion by itself is thought unlikely to give rise to a self-sustaining outbreak, it is possible that transfusion in combination with other potential routes of transmission such as surgical or invasive medical procedures could result in such an event. Confirmation of clinical transmission of variant CJD via blood transfusion led to the introduction of deferral of donors in the UK who themselves have definitely or probably received blood components in order to reduce the risk of tertiary and higher order transmissions, at the cost of a 5% to 10% loss in blood donations. However, surveys have documented considerable inconsistency in donor recall of blood transfusion, undermining confidence in the effectiveness of such measures. Donor exclusion criteria are blunt risk management tools that have the potential for significant negative impact on individual donors and on the blood and tissue supply.

### Donor Screening Assays

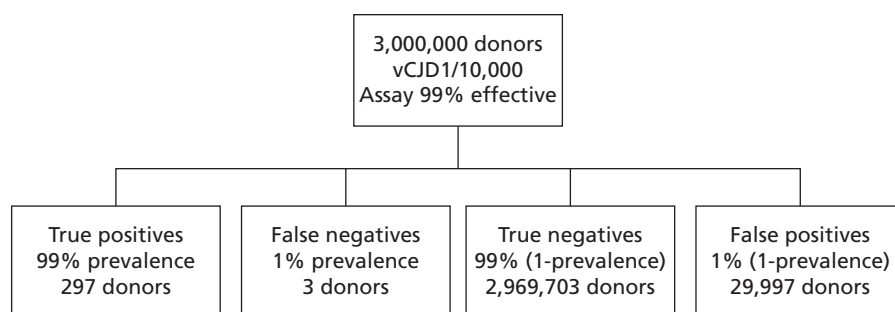
The development of peripheral blood assays aimed at the detection of subclinical CJD in donor populations is hampered by the absence of an antibody response or of detectable DNA associated with transmission of the infectious agent.

Surrogate assays providing nonspecific evidence of neurologic damage<sup>96,97</sup> or alteration in peripheral tissue physiology<sup>98,99</sup> have thus far proved insufficiently sensitive and specific for subclinical CJD.

PrP<sup>TSE</sup> detection remains the most promising route to development of a preclinical screening assay,<sup>100-101</sup> despite caveats about the precise relationship with infectivity. Achieving the required levels of sensitivity and specificity has, however, proved to be a major challenge. It has been estimated that the threshold of detection may need to be as low as 10<sup>6</sup> molecules of PrP<sup>TSE</sup>/mL in peripheral blood.<sup>102</sup> Immunoblotting with proteinase K digestion and gel electrophoresis has been enhanced using phosphotungstic acid precipitation and chemiluminescence,<sup>103</sup> but is probably still insufficiently sensitive to detect PrP<sup>TSE</sup> in blood. Capillary immunoelectrophoresis following proteinase K digestion and competitive antibody binding was the first method to describe detection of PrP<sup>TSE</sup> in blood,<sup>104</sup> but has proved complex and difficult to reproduce in other laboratories.<sup>105</sup> A number of attempts have now been made to move away from the use of proteinase K resistance as the defining principle.<sup>106</sup>

Several assays use denaturation of PrP<sup>TSE</sup> aggregates, the best characterized of which is the conformation dependent immunoassay.<sup>107</sup> Several PrP<sup>TSE</sup>-specific monoclonal antibodies have now been described, of which 15B3 is the best characterized.<sup>108</sup> Several groups have developed assays predicated on differential binding of other ligands including polyanionic compounds,<sup>109</sup> synthetic polypeptides,<sup>6</sup> streptomycin/calix-6-arene,<sup>6</sup> and palindromic peptides.<sup>110</sup> Some of these approaches are now showing sufficient sensitivity to detect infectivity in the peripheral blood of TSE-infected animals. Finally, two approaches appear to amplify PrP<sup>TSE</sup> and/or infectivity. Protein folding cyclic amplification

**Figure 50-1.** The impact of sensitivity and specificity of an assay for variant CJD on test results from a donor population.



uses repeated cycles of incubation and sonication to amplify PrP<sup>TSE</sup> up to 10<sup>7</sup>-fold,<sup>44</sup> whereas in-vitro cell culture has also proved capable of amplifying murine scrapie.<sup>111</sup> Such amplification might prove a valuable adjunct to other detection methods.

There are three key concerns relating to the introduction of donor screening assays.

1. The specificity of an assay is dependent not only on its technical properties, but also on the population in which it is deployed. An assay with a satisfactory positive predictive value in the context of patients with suspect disease may have a very poor positive predictive value in the context of general population screening. The potential impact of an assay with a low specificity on the false-positive rate of a healthy donor population is illustrated in Fig 50-1. Overall, there is a clear need for assays employing different analytical principles, one or more of which can be used for primary screening and others as supplementary or confirmatory assays to help control the false-positive rate.

2. The validation of such assays is problematic.<sup>112-114</sup> The standard approach to validation of microbiological screening assays uses large numbers of samples from patients known to have the disease in question. Patients with variant CJD in particular are fortunately rare and it will be problematic to obtain large volumes of blood for ethical and practical reasons. It is likely, therefore, that it will be necessary to carry out the development and validation of peripheral blood screening assays on spiked brain homogenates and endogenously infected animal models, raising concerns about the relevance of these models to the human setting.

3. Consideration will need to be given to the impact of introduction of a screening assay on donors. There are likely to be difficulties in the discrimination of false from true positives and, indeed, in understanding the “meaning” of a true-positive result in terms of the implications for the donor. Donors will have to be informed that they will be screened and notified of any positive result. The psychological and social impact on the individual is likely to be high and the negative impact on the blood supply could be profound. Further, detailed consideration needs to be given to the practical and ethical implications of introducing an assay and the provision of adequate supporting resources.<sup>115-117</sup>

### Blood Component Processing

Whole blood is now rarely transfused, the majority of components being collected via apheresis or derived from whole blood

donation and processed into red cell concentrate (in additive solution), platelet concentrate, and fresh frozen plasma. In addition, in the UK, Ireland, France, and some other countries, all clinical blood components are subject to universal leukocyte reduction, which removes 3-4 log<sub>10</sub> leukocytes.<sup>118-120</sup> The rodent studies of Brown et al<sup>33,34</sup> and Gregori et al<sup>36</sup> suggest that it is likely that component processing brings about some reduction in overall infectivity because of reduction in the amount of residual leukocytes and plasma, but it is unlikely that this will significantly affect transmissibility.

Several companies are working on the development of prion reduction filters for red cell components that offer the possibility of a further reduction in infectivity.<sup>121,122</sup> Current risk management models suggest that a 3 log<sub>10</sub> reduction in infectivity with use of the device would be needed in association with leukocyte reduction in order to effect a reduction in the risk of transmission. Potential detrimental effects include additional blood loss in the dead volume of the filter and the possibility of alterations to red cell antigenicity or membrane properties. There are concerns about the evaluation of the efficacy of these technologies given that they have developed using spiked brain homogenates and endogenous infectivity animal models. The UK and Irish Blood Services have developed a series of quality, efficacy, and operational specifications and are commissioning independent evaluation and clinical safety studies.

### Plasma Derivative Manufacture

It is unlikely that the virus inactivation steps used in the manufacture of plasma derivatives will have a significant impact on prion infectivity.<sup>123</sup> However, work based on consideration of the likely physicochemical characteristics of the prion agent and the partitioning effects of plasma fractionation suggests that a significant reduction in infectivity may occur during the fractionation process, resulting in a relatively low risk of transmission by plasma derivatives.<sup>124</sup> Studies of spiked brain homogenates support this prediction, but are unlikely to accurately reflect the physicochemical nature of the infectious agent in plasma.<sup>125-128</sup> A smaller number of endogenous infectivity studies have been carried out in animal models that again support the general premise, but are unable to demonstrate more than a 1- to 2-log reduction because of low concentrations of infectivity in the starting material.<sup>34</sup> Several manufacturers are using nanofiltration as a second

virus reduction step, and these filters have, at least experimentally, demonstrated prion removal, mainly using brain homogenate spikes. Prion-binding affinity chromatography systems are under development but are probably still some way from routine application. There has been no documented case of transmission of sporadic CJD by plasma derivatives. Surveillance of patients exposed to large volumes of plasma products have also not shown any evidence of transmission. Some of these products are known to have been manufactured from pools that contained plasma from a donor who subsequently developed variant CJD. Most such individuals are considered to be “potentially at risk for public health purposes.”<sup>129</sup>

### Cellular, Tissue, and Organ Transplantation

Many types of cells, tissues, and organs are transplanted.<sup>130</sup> As noted previously, the concentration of infectivity in such tissues is uncertain, although broadly speaking, a very low concentration would suffice to cause transmission of disease if the donor were infected. Deferral of transfused donors has proved to be complex because of the nature of the donor population(s), the shortage of some tissues, and the lifesaving nature of the procedure—particularly of organ transplantation. A feasibility study has been initiated to look at the possibility of testing tonsil from cadaveric donors for PrP<sup>TSE</sup> accumulation. Broadly speaking, any processing that reduces the amount of cellular material and also avoids pooling is likely to be of benefit. There are ongoing studies of the applicability of, for example, washing method for bone processing. Agents that proved effective in the decontamination of surgical instrumentation are largely inapplicable to cells or organs although some might be applicable to acellular tissues such as heart valves, bone, or tendons. Finally, it is possible that some of the compounds found to mitigate TSE infectivity in vitro and/or in vivo could be applicable to these products.<sup>131,132</sup> Substantial further research and development work will need to be carried out before results of these investigations can be translated to the clinical setting.

### Ethical, Legal, and Societal Considerations

Many of the issues raised above pose not only scientific and medical challenges, but also wider ethical, legal, and societal questions. Early uncertainty over the possibility of secondary transmission of CJD by blood transfusion or plasma derivatives led to the implementation of risk management strategies based on the precautionary principle, perhaps best articulated by the Krever Commission in Canada<sup>133,134</sup>:

“Preventive action should be taken when there is evidence that a potentially disease-causing agent is or may be blood borne, even when there is no evidence that recipients have been affected. If harm can occur, it should be assumed that it will occur. If there are no measures that will entirely prevent the harm, measures that may only partially prevent transmission should be taken.”

The accumulating evidence of the existence of a cohort of individuals subclinically infected with variant CJD prions in the UK, along with recent reports of clinical transmission by blood transfusion, speak to the prudence of these measures. However, important uncertainties persist and the effectiveness of current risk reduction measures including donor selection procedures, component processing, and plasma fractionation remains unclear. New technologies such as prion reduction filters and prion screening assays are under development and the precautionary principle would speak to the need to implement these where possible. However, such measures are not without potential countervailing risks and ethical problems.<sup>135</sup> Donor deferral criteria can be considered discriminatory, and the introduction of a donor screening assay could have a serious impact on the psychological health of individuals who test positive, leading to difficulties in balancing non-malevolence with regard to the patient against non-maleficence with regard to the donor. Donor deferral and screening measures could have a serious negative impact on the sufficiency of blood and tissue supply, leading to a failure of beneficence.<sup>136</sup> Component processing technologies such as universal leukocyte reduction<sup>137</sup> and prion reduction filters<sup>138</sup> offer the opportunity to circumvent these problems, but at significant financial cost, raising issues of equity of access to health-care resources. Finally, compulsory notification of people at potentially increased risk of exposure to CJD infection through blood components or plasma derivatives raises the issue of respect for autonomy.<sup>139</sup>

In this context, continued efforts should be made to improve the evidence base regarding clinical blood transfusion and tissue transplantation<sup>140</sup>; to promote a conservative approach to the use of human blood and tissue products<sup>141</sup>; to ensure that patients are appropriately informed<sup>142,143</sup>; and to facilitate public understanding of, and engagement with, the uncertainties and ethical dilemmas inherent in managing this risk.<sup>144</sup>

### Acknowledgments

I would like to thank Professor Bob Will, Dr. Lorna Williamson, and Dr. Patricia Hewitt for their comments on this manuscript. Any errors are the responsibility of the author alone.

### Disclaimer

The author has disclosed no conflicts of interest.

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Transfusion-transmitted infections (TTIs) involving the infectious agents described in Chapters 46–50 have been significantly reduced in industrialized countries during the last two decades by careful donor selection and extensive laboratory testing. However, blood transfusions represent an ideal portal of entry for infectious agents and often contain trace amounts of endogenous bacteria (mostly from the gut or from presymptomatic infections) and/or exogenous bacteria (from the skin). With the volumes generally collected during a donation, trace contaminations are a problem. This is particularly true for platelet products (stored at 22°C) and in the transfusion of immunocompromised patients. Important interventions to reduce the risk for trace contaminations are careful donor selection, enhanced cleansing of the venipuncture site, use of diversion pouches for the first 15 to 20 mL of a blood collection,<sup>1</sup> and the testing of platelet preparations for the presence of bacteria. Risks for TTIs are escalated by emerging infections combined with increased international air travel.<sup>2</sup> This has been demonstrated by the mosquito-spread viruses, West Nile virus in the United States and the chikungunya virus in the Indian Ocean region. For the former a nucleic acid amplification test (NAT) has been developed, while this is not the case for chikungunya virus, which spread to Italy in 2007 and posed a risk in the Mediterranean area. On the Île de Réunion in the Indian Ocean in 2006, more than 25% of the inhabitants were infected. As a temporary measure in order to ensure a safe blood supply, components were supplied from France [except platelets, which were prepared locally by apheresis and subjected to pathogen inactivation (PI)].<sup>3</sup>

Programs for careful donor selection and extensive laboratory testing pose both organizational and economic challenges in most developing countries. In addition, the epidemiology is less favorable than in most developed countries, the use of whole blood is still dominating, and safe fractionated plasma proteins

are mostly unavailable. Therefore, TTIs are a serious problem, causing thousands of infections a year particularly with hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), and malaria. Robust and affordable PI technologies for whole blood and blood components would be of great value in these countries. In developed countries, the risk for bacterial and protozoan infections and emerging infectious agents are the main drive for PI.

Several approaches have been explored for PI. Except for solvent/detergent (SD) treatment, all are based on PI of single plasma units from recovered or apheresis plasma. Whether applied under good manufacturing practice (GMP) conditions in blood centers or on a larger scale outside blood centers, in-process control of single plasma units is not performed. Thus, exceptionally high loads of a pathogen could slip through; NAT should be performed in spite of PI.<sup>2</sup>

Pathogen inactivation of pooled fractionated plasma proteins has virtually eliminated the risk of TTIs, without compromising the quality of the products significantly. After addition of stabilizers, some proteins such as albumin have been pasteurized with excellent pathogen safety records. However, it took almost 60 years before attention was drawn to the fact that the addition of caprylic acid and N-acetyl-DL-tryptophan as stabilizers during pasteurization impairs drug binding of pharmaceutical grade albumin.<sup>4</sup>

In this chapter the much more challenging PI of blood components are discussed. Solvent/detergent treatment, which is successfully applied for biopharmaceutical plasma products, dissolves cell membranes; hence, it is highly effective also against intracellular pathogens but not applicable for cellular blood components. PI methods that target nucleic acids have been the prime choice for PI of cellular components, but their efficiency against intracellular microorganisms depends on the ability to penetrate cell membranes.

PI of blood components should inactivate or remove all types of infectious agents, without inducing neoantigens or reducing the function or life span of a blood component. PI should not result in residual toxic substances or involve a risk greater than any TTI associated with the original blood component.<sup>5</sup> Because

toxicity may first be revealed after large-scale clinical use, Phase IV postmarketing studies and hemovigilance programs are important.

Plasma Pathogen Inactivation

Solvent/Detergent Treatment

SD treatment is the most extensively used and best validated PI technology: 50 to 60 million doses of SD-treated pooled fractionated plasma proteins have been given without the reported transmission of enveloped viruses. However, the method does not inactivate nonenveloped viruses, and nanofiltration or heat treatment has been introduced as a second PI step for many fractionated plasma proteins. SD treatment removes infectivity by disrupting the membranes of enveloped viruses; bacteria, protozoa, and cells are also affected, but so far this has not been extensively validated.<sup>2</sup> The most frequently used technique is the combination of 1% tri-(N-butyl)-phosphate (TNBP) and 1% polyoxyethylene-p-t-octylphenol (Triton X-100) for 4 hours at 30 to 31°C.<sup>6</sup> TNBP acts as an organic solvent and removes lipids

from the membranes; it is used alone in some protocols for PI. Triton X-100 is a non-ionic detergent that disrupts lipid bilayers for easier extraction of lipids; it also stabilizes TNBP.<sup>6</sup> SD treatment was first licensed for clotting factors in 1985 and has been in use for the treatment of plasma since 1991.<sup>7,8</sup> The inactivation rate of enveloped viruses is fast and the safety of the method very high (Table 51-1).<sup>7,8</sup> Inactivation is below virus detection levels (>6log) within 15 minutes,<sup>9</sup> except for vaccinia virus, which shows a relative resistance, but is inactivated with TNBP and Triton X-100 according to the protocol mentioned above.<sup>10</sup> So far, all tested enveloped viruses, including the recently emerging ones, are inactivated. For plasma no second PI step is available, but immune antibodies in a plasma pool can protect against disease with hepatitis A virus (HAV) and parvovirus B19.<sup>11,12</sup> In addition, NAT screening for HAV and parvovirus B19 ensures the safety of SD-treated plasma, as an essential regulatory requirement.

A major advantage of SD treatment is that the inactivation reagents in general do not affect proteins and protein activity in plasma. One important exception is reduction of the α<sub>2</sub>-antiplasmin activity by 70% to 80% during SD treatment. This seems to

Table 51-1. Inactivation Agents, Susceptible Pathogens, and Modified Targets

| Inactivation Agent            | Susceptible Organism                                                                                                                                                                                                    | Target/Modification                                            |
|-------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------|
| Solvent/Detergent             | <ul style="list-style-type: none"><li>• Enveloped viruses</li><li>• Intracellular viruses</li></ul>                                                                                                                     | Lipid envelope solubilization                                  |
| Methylene blue plus light     | <ul style="list-style-type: none"><li>• Enveloped viruses</li><li>• Some nonenveloped viruses</li></ul>                                                                                                                 | Free radical<br>Guanidine                                      |
| S-59 plus ultraviolet A light | <ul style="list-style-type: none"><li>• Enveloped viruses</li><li>• Intracellular viruses</li><li>• Some nonenveloped viruses</li><li>• Bacteria not including spores</li><li>• Protozoa</li><li>• Leukocytes</li></ul> | Pyrimidine adducts and cross-links                             |
| Riboflavin plus light         | <ul style="list-style-type: none"><li>• Enveloped viruses</li><li>• Intracellular viruses</li><li>• Some nonenveloped viruses</li><li>• Bacteria (spores unknown)</li><li>• Protozoa</li><li>• Leukocytes</li></ul>     | Guanidine oxidation and possible adducts to thymine and adenin |
| S-303                         | <ul style="list-style-type: none"><li>• Enveloped viruses</li><li>• Intracellular viruses</li><li>• Some nonenveloped viruses</li><li>• Bacteria (spores unknown)</li><li>• Protozoa</li><li>• Leukocytes</li></ul>     | Nucleic acid adducts                                           |
| PEN 110                       | <ul style="list-style-type: none"><li>• Enveloped viruses</li><li>• Intracellular viruses</li><li>• Some nonenveloped viruses</li><li>• Bacteria (spores unknown)</li><li>• Protozoa</li><li>• Leukocytes</li></ul>     | Guanine adducts                                                |

be caused by Triton X-100 because a reduction of only about 10% is observed with Triton X-45, which in preliminary virus inactivation validation is as effective as Triton X-100.<sup>13</sup> Triton X-45 and X-100 differ in molecular mass, the former having only five ethylene oxide groups, the latter nine or 10 groups. This difference in molecular mass suggests a differential impact of the two non-ionic detergents on the conformation (and activity) of  $\alpha_2$ -antiplasmin.<sup>13</sup> After removal with oil extraction and hydrophobic interaction chromatography, TNBP and Triton X-100 are either undetectable or present as trace contaminations far below toxicity levels.<sup>6</sup>

SD treatment is the only current technology for PI of pooled plasma. This allows for the production of a standardized biopharmaceutical product with extensive in-process control and without the significant normal variation in plasma protein concentrations observed in single plasma units. Pooling also dilutes and in some instances neutralizes antibodies and allergens, and allows the production of ABO-independent universal plasma (see later). There is a risk that emerging TTIs would be increased by pooling. However, SD plasma pools (60–380 L) are 10 to 100 times smaller than those used in the production of PI fractionated plasma proteins. Recently, a very gentle method for SD treatment of single plasma units and cryoprecipitate at blood centers was published.<sup>14,15</sup>

SD-treated pooled Fresh Frozen Plasma (SDPP) was introduced in Europe in 1991, with Octaplas (Octapharma AG, Lachen, Switzerland) as the leading product. Octaplas is a licensed biopharmaceutical product in 29 countries worldwide (including Canada and Mexico in North America). A total of over 6 million units of SDPP have been transfused so far. In the United States, SDPP (PLAS+SD, Vitex, Watertown, MA) was licensed in the second half of the 1990s but later withdrawn from the market.<sup>2</sup> After SD treatment, these SDPP products are normally frozen in 200-mL plastic bags; however, the South African Bioplasma FDP is lyophilized.

Nine studies covering all indications for plasma (including six European prospective randomized trials with SDPP) have investigated the clinical efficacy and tolerance, with Octaplas as the best-documented product.<sup>16,17</sup> Although the SD treatment process has been the same for all SDPP products, there have been some differences in the quality of plasma and manufacturing processes.<sup>16,17</sup> Apheresis plasma is preferred, followed by recovered plasma optimally frozen within few hours after collection. Plasma from whole blood stored for 15 hours before separation and freezing has a reduced content of coagulation factors and inhibitors.<sup>18</sup> The usage pattern of such plasma and some differences in production processes could account for the thromboembolism and compositional differences observed with PLAS+SD but not with Octaplas.<sup>19–21</sup> All SDPP products have low levels of  $\alpha_2$ -antiplasmin. Except for protein S in the lower normal range and the reduction of  $\alpha_2$ -antiplasmin, Octaplas has a concentration of coagulation factors and inhibitors and clinical effect similar to apheresis FFP (frozen within 24 hours after collection).<sup>17,22</sup> SD treatment of single plasma units (1% TNBP + 1% Triton X-45 or only 2% TNBP) has been reported to have minimal effect on coagulation factors

and inhibitors, including protein S and  $\alpha_2$ -antiplasmin.<sup>15</sup> This promising method, however, needs further validation.

$\alpha_2$ -antiplasmin is an acute-phase serine protease inhibitor of plasmin that is synthesized in the liver.<sup>23</sup> This explains why most clinical observations show that low  $\alpha_2$ -antiplasmin levels in SDPP are insignificant except in patients with liver deficiency or fibrinolysis.<sup>17</sup> In these patients, antifibrinolytic treatment or administration of the serine protease inhibitor aprotinin has to be considered.<sup>2,24</sup> In an enhanced version of Octaplas  $\alpha_2$ -antiplasmin levels are now reduced by only 30% to 40% (T-E Svae, Octapharma, personal communication). Low levels of protein S have been associated with thromboembolism observed in liver transplantation with PLAS+SD,<sup>17</sup> and a retrospective study in Europe indicated possible thromboembolism after repeated plasma exchanges with Octaplas in patients with thrombotic thrombocytopenic purpura (TTP).<sup>25</sup> However, in a large study, the same authors subsequently observed no problems with venous thromboembolism and concluded that there was no difference in the number of exchanges needed when cryosupernatant and Octaplas were compared. In addition, they reported that allergic/urticarial and citrate reactions were more common with cryosupernatant.<sup>26</sup>

Adverse events are less common with SDPP than with ordinary FFP. Particularly important is that there have been no reports of transfusion-related acute lung injury (TRALI) after transfusion of over 5 million units of SDPP in Europe.<sup>27,28</sup> Hemovigilance data indicate that febrile, allergic, or anaphylactic reactions are reduced by 70% to 80% with SDPP.<sup>21</sup> These observations are best explained by dilution/neutralization that results from pooling and removal of all cellular components during the SD treatment process.

An improved version of SDPP is ABO-universal plasma that eliminates the need for ABO group-specific plasma.<sup>2</sup> It is based on the principle that anti-A and anti-B can be neutralized by soluble A and B antigens in plasma. This was first exploited during World War II, when the Allied Forces used randomly pooled plasma. Such a lyophilized product was licensed for emergency needs in several European countries until the 1970s, but represented a high risk of transfusion-transmitted hepatitis.<sup>2</sup> Since 1996, a lyophilized ABO-universal SDPP, Bioplasma FDP (National Bioproducts Institute, Pinetown, South Africa), has been prepared from low-titered plasma pools in South Africa.<sup>29</sup> More than 370,000 units have been transfused so far, with an excellent record of safety and efficacy.<sup>2</sup> In Europe an ABO-independent universal SDPP has been developed by Octapharma by proportionally pooling A, B, and AB plasma. The product (tentatively named Uniplas) is not yet licensed, but has been used successfully in clinical trials in cardiac surgery and liver resection in Europe.<sup>30–32</sup>

## Photosensitizers

### Methylene Blue Light Treatment

Methylene blue (MB) is a positively charged phenothiazine dye with high affinity for negatively charged compounds, such as guanine, proteins, and some lipids. The virus inactivating activity of illuminated phenothiazines has been recognized since the

early 1930s.<sup>33</sup> Evidence suggests that MB interacts with nucleic acids through intercalative binding and produces singlet oxygen-mediated damage, primarily to nucleic acid guanosine residues, upon illumination with visible light.<sup>34</sup> MB light treatment is effective against enveloped and some nonenveloped viruses (Table 51-1). MB does not effectively permeate plasma membranes; thus intracellular pathogens and leukocytes may not be fully inactivated. Therefore, the original protocol developed by Institute Springe introduced a freeze-thaw step to destroy residual leukocytes. Grifols (Barcelona, Spain) employs leukocyte filtration of blood, which also removes platelets and to some degree cellular fragments and microparticles, and brings residual leukocyte counts in plasma well below  $1 \times 10^6$ .<sup>35</sup> However, only plasma membrane filters remove all cellular components, and reduce intracellular HIV infectivity to detection limits after MB light treatment of plasma. Binding of MB to membrane proteins may affect red cell viability, while binding to plasma proteins reduces coagulation factor activity in plasma, cryoprecipitate, and cryosupernatant.<sup>37</sup> Toxicity of MB is low. Intravenous doses of 1 to 5 mg/kg have been administered in clinical situations without the report of serious adverse effects; a unit of MB-treated plasma would represent 0.0012 mg/kg for an adult, and could be reduced to 0.00012 mg/kg with absorption devices.<sup>2</sup> No neoantigenicity caused by various intercalated proteins remaining after MB treatment have been reported. However, on theoretical basis, long-term side effects cannot be ruled out completely because of the binding of MB to proteins.<sup>21</sup>

MB-treated plasma is the second most frequently used pathogen-inactivated FFP, and more than 4 million units have been used clinically in European countries, including Germany, Belgium, France, Greece, Italy, Spain, Switzerland, and the UK. The method is applied on single plasma units. The original Springe protocol was factory based, and in use for more than a decade in Germany and Switzerland.<sup>38</sup> Grifols has introduced a similar factory-based method, but with leukocyte filtration instead of a freeze-thaw step. This method is still in use in Spain. Macopharma (Tourcoing, France) has modified MB treatment of plasma for use in blood centers, and improved the method. The firm has introduced the Theraflex MB-Plasma system, with a 0.65-micron plasma filter and a removal device for MB that eliminates more than 90% MB and its photoproducts, without any significant additional loss of coagulation factors.<sup>2</sup> The system is granted Conformité Européenne (CE) mark and used to prepare PI plasma in house at a number of blood centers in Europe. In Germany the Paul Ehrlich Institute (Frankfurt, Germany) has granted authorization for manufacture and sale of plasma treated with the Theraflex MB-Plasma system.

In spite of extensive use, there have been no full reports of large randomized trials with MB-treated plasma using relevant endpoints such as blood loss or exposure to other blood components.<sup>39</sup> In one small study, cardiac surgery patients were transfused with either MB-treated plasma or SDPP. The former gave better replacement of protein S and  $\alpha_2$ -antiplasmin, but no difference in blood loss.<sup>40</sup> A 5-year observational study in Greece

with the Theraflex MB-Plasma system reported good safety and a reduced number of adverse events.<sup>41</sup>

As with all other PI methods, MB treatment reduces the potency of some active elements of plasma. Fibrinogen, Factor V, and Factor VIII activity are decreased by approximately 30%; the effect on other coagulation factors and inhibitors is smaller.<sup>2,41</sup> Significant prolongation of fibrin polymerization time has been reported; the prolongation is, however, eliminated after admixture of 60% Octaplas.<sup>42</sup> Thrombin generation capacity is impaired in MB-treated plasma.<sup>43</sup> Overall, satisfactory clinical results and efficacy have been reported with MB-treated plasma. However, it seems to be less effective than FFP as replacement fluid in plasma exchange for TTP, in spite of normal levels of von Willebrand factor cleaving enzyme (ADAMTS13) in MB-treated plasma.<sup>2,44</sup>

### Psoralen Ultraviolet Light Treatment

Furocoumarins, which include psoralens, are active compounds isolated from plants; they have been known as photosensitizers since ancient times. Amotosalen hydrochloric acid (also known as S-59) is a synthetic psoralen (Fig 51-1) specially selected by Cerus (Concord, CA) for PI of blood components because it crosses plasma membranes efficiently and demonstrates excellent protection against pathogens.<sup>6</sup> S-59 is activated by ultraviolet A (UVA) light. Because hemoglobin absorbs UVA light, S-59 is not applicable for PI of red cells.

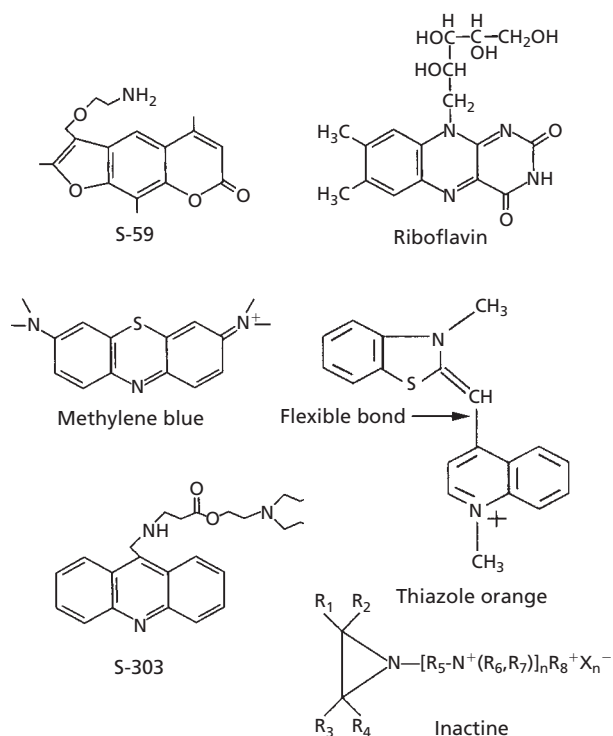


**Figure 51-1.** Structures and general formula for some pathogen inactivating agents. The formula for Thiazole orange is used with permission from Skripchenko et al.<sup>45</sup>



S-59 targets the helical region of single- or double-stranded DNA or RNA, and intercalates almost instantly. Upon exposure to UVA light, S-59 binds covalently to pyrimidine, and with continued UVA light exposure a second bond is generated cross-linking double-stranded structures, while single-stranded structures are cross-linked in loops. S-59 photodegrades into well-characterized molecules that are rapidly excreted.<sup>6</sup> In addition to targeting nucleic acids, S-59 also binds to lipids and proteins. Thus, about 15% of the initially added S-59 remains bound to plasma and platelets even after removal of S-59 and its photoproducts with a compound absorbing device (CAD). Most S-59 is associated with lipids, but 1% to 2% is associated with proteins.<sup>2</sup> Psoralen UVA light treatment with S-59 inactivates a broad spectrum of enveloped viruses, bacteria, protozoa, and residual leukocytes, but its effect on nonenveloped viruses is more variable (Table 51-1).<sup>6,46</sup>

The INTERCEPT Blood System device developed by Cerus uses S-59, UVA illumination, and a CAD for the removal of residual S-59 and metabolites. In Europe the system was granted CE mark for PI of plasma in 2006, and is supposed to be implemented under GMP conditions in blood centers. In the United States it is approved only for clinical trials by the FDA. Preclinical studies have demonstrated high levels of safety.<sup>47,48</sup> Fibrinogen, Factor V, Factor VII, Factor VIII, and Factor X are reduced by 17% to 30%, while coagulation factor inhibitor activity is less affected.<sup>46</sup> Four randomized studies indicate adequate clinical effect, including support of plasma exchange for TTP<sup>48-51</sup>; however, more than 60% of the patients with congenital coagulation deficiencies experienced urticaria.<sup>49</sup> Although no neoantigenicity or toxicologically relevant effects have been observed with INTERCEPT-treated plasma, Phase IV postmarketing studies could still reveal such effects resulting from some binding of S-59 to lipids and proteins.

### Riboflavin Light Treatment

In spite of phototoxic targeting of nucleic acids, vitamin B2 (riboflavin) is generally recognized as safe by the US FDA. This safety is supported by extensive experience with phototherapy treatment of neonatal jaundice (see Chapter 27). Riboflavin and its photoproducts are present in a wide range of foods and natural products in common use. In addition, riboflavin, its photoproducts, and its catabolites are detectable in normal blood, which suggests that their presence may be ubiquitous.<sup>52</sup> However, further research is required because the level of photoproducts is considerable higher in riboflavin-treated blood components. Both oxygen-dependent (formation of free radicals) and oxygen-independent (electron transfer) processes are involved in the phototoxic PI effect of riboflavin, which damages guanine and may form adducts of thymine and adenine.<sup>53</sup> Riboflavin light treatment has proven effective against a range of pathogens, including bacteria, enveloped viruses, protozoa, leukocytes, and some nonenveloped viruses (Table 51-1).<sup>52</sup>

Navigant Biotechnologies (Lakewood, CO) has developed the Mirasol system for riboflavin treatment of blood components.

Because riboflavin can be photoactivated by visible as well as UV light, the Mirasol system is being adapted for PI for all blood components, including red cells and whole blood. The system is similar to INTERCEPT, except that removal of reagents may not be necessary, and is not currently performed. Mirasol has been adapted for PI of platelets and plasma, while treatment of red cells and whole blood still is under development. Extensive PI of selected viruses (including parvovirus), bacteria, and protozoa in addition to leukocyte inactivation has been reported in platelet concentrates.<sup>52</sup> Similar results have been obtained with Mirasol treatment of FFP (RP Goodrich, Navigant Biotechnologies, personal communication).

Compared with the INTERCEPT system, the Mirasol system for PI of plasma reduces the time plasma is stored in liquid state, and early studies indicate that plasma protein activity is only marginally affected.<sup>52</sup> Preclinical studies with Mirasol-treated plasma are promising, and no neoantigenicity has been observed. Pending the successful completion of remaining field verification and clinical studies in Europe, the Mirasol system for PI of plasma is expected to receive a CE mark (RP Goodrich, Navigant Biotechnologies, personal communication).

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## Platelet Pathogen Inactivation

### Psoralen UV Light Treatment

The INTERCEPT Blood Systems device for PI of platelets is similar to the one for plasma, and was granted CE mark in Europe in 2002; the FDA so far has approved it only for clinical trials. During the rather complex operation of PI with INTERCEPT, platelets come in contact with many surfaces that may induce surface changes and loss of granule contents. Studies have demonstrated inactivation of a broad spectrum of viruses, bacteria, protozoa, and leukocytes, including clinically relevant nonenveloped viruses such as parvovirus B19 (Table 51-1).<sup>6</sup> Typical experiments have been reported by Lin et al.<sup>54</sup> INTERCEPT treatment also inactivates mitochondrial DNA and inhibits cytokine synthesis, while platelet metabolic functions seem retained.<sup>55</sup> In-vitro INTERCEPT-treated platelets demonstrate increased CD61 microparticle formation, higher metabolic rate, accelerated metabolic changes, and reduced agonist-induced aggregation responses. Spontaneous platelet activation measured by fluorescent-activated cell sorter showed increased expression of CD62P and CD42b after INTERCEPT treatment; in addition, phosphatidyl-serine (PS) on the platelet surface (a hallmark of apoptosis) is significantly increased.<sup>56-58</sup> These changes are accelerated when the storage time is increased to 7 days. To what degree the formation of apoptotic cells, as measured by PS exposure, is of clinical significance remains to be established. In-vitro data show cytokine accumulation also in INTERCEPT-treated platelet concentrates.<sup>56</sup>

A European study including 166 thrombocytopenic patients transfused with INTERCEPT-treated buffy-coat platelets showed comparable platelet count increments and adverse reactions to untreated platelets.<sup>59</sup> However, in a larger US study (n = 645)

assessing apheresis platelets, the 1-hour posttransfusion corrected count increment and average number of days to next platelet transfusion were slightly reduced, while the number of platelet transfusions was increased compared to untreated platelets.<sup>60</sup> In these studies storage time was limited to 5 days. Many facilities have increased storage time to 7 days without additional clinical trials in spite of the in-vitro study results. After CE marking in 2002 the system has been used for the production of more than 50,000 doses of PI platelets for routine clinical use in 14 European countries, and is reported to have replaced bacterial testing, gamma irradiation, and the use of cytomegalovirus-negative platelets. In addition there has been a report of a reduction in allergic nonhemolytic transfusion reactions with INTERCEPT-treated platelets.<sup>2</sup> Although no neoantigenicity or toxicologically relevant effects have so far been observed after clinical use of INTERCEPT-treated platelets, Phase IV postmarketing studies could reveal such effects resulting from some binding of S-59 to platelet lipids and proteins.

### Riboflavin-Treated Platelets

The Mirasol system for PI of platelets is similar to the one for plasma. It is less complex than the INTERCEPT system in that removal of reagents is not performed; thus it may be more gentle to the platelets. Nevertheless, this PI process also leads to some platelet activation as expressed by increased metabolic activity and P-selectin expression.<sup>53</sup> However, both in-vitro activity and mitochondria function seem not to be affected.<sup>61,62</sup> Extensive PI of selected viruses (including parvovirus B19), bacteria, and protozoa and inactivation of leukocytes has been reported.<sup>53</sup> Preclinical studies have demonstrated that in-vitro and in-vivo capabilities of Mirasol-treated platelets are similar to INTERCEPT-treated platelets.<sup>53,61,63</sup> After a Phase III study was completed in Europe in 2007, the Mirasol system for platelet PI was granted CE marking for application under GMP conditions in blood centers.

### Thionine Light Treatment

The phenothiazine dye thionine and UV light combined with strong agitation is effective in the PI of platelets, and development of a protocol is being pursued by Blood Centers of the German Red Cross and MacoPharma (H Mohr, Institute Springe, Germany, personal communication). However, strong agitation could pose problems with irreversible activation of platelets.

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## Red Cell Pathogen Inactivation

PI systems for red cells and whole blood have been explored with some negative results but also some promising developments.

### Alkylating Agents

#### FRALE Compounds

Frangible anchor linker effector (FRALE) compounds are alkylating agents that specifically inactivate DNA/RNA via

covalent adducts to the nucleic acid. The compounds consist of a nucleic acid-binding ligand that serves as an anchor and promotes nucleic acid specificity, an alkylating agent that functions as an effector, and a chain that joins the two moieties.<sup>6,64</sup> Cerus has developed the Helinx system for PI of red cells with S-303 (Fig 51-1), which is a compound with an acridine-based anchor. S-303 is designed so that the alkyl chain is cleaved upon contact with blood at a rate slower than the rate of binding and alkylation to nucleic acids. This does not inhibit PI, but promotes the breakdown of residual unreacted compound into S-300, which does not further interact with nucleic acids.<sup>6,64</sup> Acridine nitrogen mustards have been shown to alkylate proteins in addition to nucleic acids.<sup>65</sup> Helinx treatment is performed at room temperature for 12 hours followed by incubation with a CAD in the final storage container for 8 hours in order to remove residual S-303 and S-300.<sup>6</sup> Helinx PI has been shown to inactivate viruses, bacteria, protozoa, and leukocytes efficiently (Table 51-1), and extensive toxicology studies indicated that the process was safe.<sup>66</sup> However, the literature with regard to the technology is sparse and mostly based on abstracts.<sup>6</sup>

PI with FRALE compounds has been applied to red cells, and the only available protocol is Helinx with S-303. After satisfactory results with in-vitro studies and genotoxicity tests, successful Phase I and II trials were performed, followed by Phase III trials in cardiac surgery and sickle cell/thalassemia patients. S-303 PI and conventional red cells were equivalent with regard to supporting the transfusion needs of cardiac surgery patients.<sup>67</sup> Nevertheless, the Phase III trial was put on hold because some patients in each group developed antibodies reactive with S-303-treated red cells, but no hemolysis.<sup>67</sup> Neoantigen formation may have been caused by alkylation of proteins by acridine nitrogen mustards.<sup>65</sup> Some patients even demonstrated antibodies before transfusion with S-303-treated red cells. These antibodies could have been induced by S-303-like agents such as the local anesthetic quinacrine, which also can alkylate proteins. Experiments to modify the Helinx system by adding quenchers has so far eliminated immunoreactivity of S-303 PI red cells,<sup>68</sup> but has not totally eliminated the formation of modified groups when red cells are tested with sensitive assays after treatment with S-303.<sup>2</sup>

### Aziridine Compounds

Aziridine compounds, such as ethylenimine, have been used to inactivate vaccines for the past 30 years. The method inactivates both enveloped and nonenveloped viruses, including members of the *Parvoviridae* family, which is often difficult to accomplish.<sup>69</sup> By forming oligomers of ethylenimine the selectivity of aziridines for nucleic acids is improved. Inactines are small molecules with a cationic tail, conferring DNA binding and an effector group based on ethylenimine or aziridine. PEN110 is a small, highly water-soluble cation with an ethylenimine oligomer (Fig 51-1), and selective affinity for nucleic acids,<sup>6</sup> but can also alkylate proteins.<sup>70</sup> It diffuses readily through cell membranes and, when forming ionic bounds with nucleic acids, the molecule is activated. The active form can then alkylate guanine. This

induces a break and results in a stop message. Vitex has developed the INACTINE system with PEN110 for PI of red cells. The treatment is performed at room temperature for 6 to 24 hours, followed by washing with unbuffered saline to levels below the limit of detection of PEN110.<sup>71</sup> Extensive studies with selected enveloped and nonenveloped viruses, bacteria, protozoa, and leukocytes (Table 51-1) demonstrate the high PI efficiency of PEN110.<sup>6</sup> Toxicology studies indicated that even if an individual human received multiple PI red cell components at once or over time, the dose would be far less than that required to have reproductive effects or induce genotoxicity.<sup>71</sup> After preclinical tests, successful Phase I and II trials were performed, but Phase III trials were stopped because of antibody responses to PEN110-treated red cells. In spite of protocol modifications the problem was not solved, and in June 2005 Vitex discontinued further investment in the development of PI with INACTINE.

### Photosensitizers

PI of red cells with photosensitizers poses problems because of light absorption by hemoglobin and the high number of red cells, which requires complex technical adjustments to secure uniform distribution of light for the treatment of Red Blood Cell (RBC) units (see Chapter 4).

Several studies have been conducted with porphyrin and phenothiazine dyes, which are rigid planar aromatic compounds activated by visible light, while psoralens are not applicable because hemoglobin absorbs UV light. However, because of hemolysis following PI of red cells with these aromatic dyes, the methods have been abandoned. Some of the hemolysis upon illumination arises from dye bound to the red cell membrane, while other damage is caused by singlet oxygen produced by illumination of dye residing in the cytoplasm or supernatant, and reacting with membrane lipids or proteins.<sup>72</sup> A new approach was opened by the discovery of flexible dyes that can act as photosensitizers when rigidly bound to substrate but cannot generate reactive oxygen species when in solution because the energy from absorbed light is dissipated as heat. Wagner and associates have pioneered the work with PI of red cells with two flexible dyes.<sup>45,72</sup>

Thiopyrylium (TP) is a flexible photosensitizer that intercalates with nucleic acids but also binds to red cells. Dipyrromethane (DP) (vasodilator, antioxidant, red cell band 3 ligand) acts as a competitive inhibitor of TP binding to red cells, and a protocol for PI of red cells was therefore designed with TP and DP. This method inactivated model viruses including intracellular HIV >6log, and six bacterial species >5log. Hemolysis increased within acceptable levels and most, but not all, red cell properties were retained after 42 days of storage.<sup>72</sup>

Thiazole orange (TO) (Fig 51-1) is another flexible nucleic acid intercalating dye, which shows less binding to red cells. Without addition of quenchers or competitive inhibitors, five tested viruses (including intracellular HIV) were inactivated >5.4log, and eight tested bacteria were inactivated from 2.3 to >7log. The PI red cells exhibited only slightly increased hemolysis, moderately elevated potassium efflux, and similar

levels of adenosine triphosphate compared to controls.<sup>45</sup> However, much work remains to understand and improve the technique, perform toxicology studies, characterize in-vitro and in-vivo properties of TO PI red cells, and eventually scale up the technique to treat entire RBC units in order to perform clinical trials. Because of some binding of TO to red cells, this dye has an inherent risk of neoantigen formation with subsequent immunization after transfusion.

### Riboflavin Light Treatment

Preliminary studies show promising results with PI of red cells using the Mirasol system adapted for red cells. Pathogen activation of microorganisms and leukocytes is similar to Mirasol treatment of platelets and plasma, while hemolysis and in-vitro red cell properties are within acceptable limits (RP Goodrich, Navigant Biotechnologies, personal communication).

The Mirasol system has now also been adapted for PI of whole blood, which afterwards can be separated into red cells, platelets, and plasma. Studies with this modified protocol include a full panel of in-vitro evaluations with the three separated blood components during storage, and radiolabeled red cell recovery and survival in healthy volunteers after 42 days of storage (RP Goodrich, Navigant Biotechnologies, personal communication). Because the chemistry employed by the Mirasol system does not involve covalent linking or alkylation and riboflavin is a natural constituent in human bodies, the risk that altered proteins in plasma or on platelet and red cell surfaces will induce antibody formation against neoantigens is small. Further studies with Mirasol PI of whole blood are, therefore, of great interest.

### Emerging Technologies

CryoFacets (Raleigh, NC) has under development an integrated system for PI, reduction of noninfectious complications, "fractionation of plasma," and effective, gentle cell processing. PI of this integrated system is based on the following:

- Vacuum ultrasound removal of oxygen in plasma or suspending solutions in order to eliminate oxidative damage of plasma proteins or cell membranes
- UVC illumination
- Ozone treatment

Plasma is first vacuum treated, then exposed to UVC light, and finally mixed with ozone. Following PI, plasma is separated into three fractions according to molecular weight: "above albumin," "albumin," and "below albumin."

Platelets are highly purified by separation with a specially developed counterflow centrifugal elutriation system; plasma, cell fragments, and bacteria are washed away. After isolation, platelets are mixed with an ozone solution in order to inactivate remaining bacteria. After a brief exposure to ozone the platelets are suspended in the "below albumin" fraction for storage. When platelets are needed the product is washed with degassed saline, which removes metabolic byproducts and cell fragments, and prepares the platelets for UVC exposure in order to inactivate viruses and possibly leukocytes. Because UVC-induced damage

to the platelets is first observed after several days, this treatment is undertaken shortly before transfusion.

Red cell treatment follows essentially the same process as that used for platelets.

PI with the CryoFacets protocols is reported to be very efficient and, because of innovative technologies, processing time is low. However, considerable in-vitro and in-vivo characterization of the PI products will be required before applicability assessments in hospitals and clinical trials can be planned.

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## Prion Elimination

None of the above-mentioned PI technologies eliminate prions. However, a significant reduction in infectivity occurs during plasma fractionation, resulting in relatively low risk for prion transmission with fractionated plasma proteins (see Chapter 50).<sup>73</sup> For SDPP (Octaplas) a reduction of 2.5 log has been reported.<sup>73</sup> Phenothiazines may react with prions,<sup>74</sup> but there are no reports on a possible effect of MB treatment of plasma. Several companies are developing prion reduction filters for red cell components and interesting studies are in progress with ligand chromatography, which may reduce prion infectivity of plasma significantly (see Chapter 50). After introduction of such ligand chromatography in the production process of Octaplas an additional reduction of at least 3 log is achieved (T-E Svae, Octapharma, personal communication).

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## Summary

SDPP is the most extensively used and documented PI plasma. An ABO-universal variant, introduced in 1996 in South Africa, eliminates the need for ABO group-specific plasma. Photochemical PI of plasma with MB light treatment has gained extensive use in Europe in spite of sparse published documentation; one protocol has gained CE mark. The protocol for psoralen light treatment of FFP gained CE mark in 2006, while the protocol for riboflavin light treatment of FFP is in advanced preclinical and clinical development. Photochemical PI of platelets with psoralen light treatment gained CE mark in 2002, and so far >50,000 platelet units have been transfused in Europe. The protocol for riboflavin light treatment of platelets gained CE mark in 2007, and is introduced in clinical settings in Europe. Two PI methods for red cells employing alkylating agents reached Phase III clinical trials; however, formation of antibodies against neoantigens on red cells has halted further progress. Riboflavin light treatment of red cells has now been adapted for PI of whole blood, which subsequently could be separated into plasma, platelets, and red cells. It is the only protocol for whole blood; therefore, further development will be followed with particular interest.

Clinical experience has shown reduced frequency (40%-80%) of allergic/immunologic (febrile nonhemolytic) adverse events with SDPP, MB-treated FFP (PI with the CE marked system), and psoralen light-treated platelets. This may be explained by the elimination of cellular components in the plasma and additional

dilution/neutralization in SDPP. Leukocyte activation and cytokine production are blocked in the platelets. In addition, no cases of TRALI have been documented after extensive use of SDPP; this may be the result of dilution and possible neutralization of leukocyte-reactive antibodies by pooling of FFP. For biopharmaceutical SDPP, the reduction of 2.5 log in prion infectivity is enhanced by ligand chromatography.

Cost-effectiveness of PI is rather low in most developed countries when compared to other strategies to reduce noninfectious risks of transfusion,<sup>75</sup> but comparable to NAT of blood components. When only the effect of virus inactivation is considered, the cost of a quality-adjusted life year has been calculated to approach US\$10 million for SDPP, and is assumed to be similar for PI of plasma by other methods.<sup>2</sup> The high cost is due to very low residual risk for recognized viral infections. During outbreaks of emerging infections and in developing countries with high residual infection risk the cost-effectiveness is much better. When TRALI is taken into consideration, the cost for SDPP is reduced to US\$40,000-170,000 per life year saved depending on patient age.<sup>2</sup> Further reduction is obtained when the reduction in allergic/anaphylactic adverse events and the benefits of ABO-universal SDPP are also considered.<sup>21</sup> For PI of platelets, no cost-effectiveness data have been published, but it has been suggested that the cost per life year saved in immunocompromised patients may be in the same range as for SD treated plasma, assuming that TRALI and allergic/anaphylactic adverse events are taken into account.<sup>16</sup> A possible introduction of riboflavin light treatment of whole blood may be expected to have considerable cost implications for all countries.

A consensus conference<sup>76</sup> recently concluded that cost-effectiveness studies should be carried out by public agencies before general introduction of a PI method and that an additional cost consequence analysis<sup>77</sup> should be included. The conference also pointed out that: "Large adequately powered randomized clinical trials should be performed to evaluate and/or confirm the effectiveness of any new PI. Postlicensure Phase VI studies should be integrated with hemovigilance systems to enhance the ability to detect adverse events."<sup>76</sup>

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## Disclaimer

The authors have disclosed no conflicts of interest.

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52 Hemolytic Transfusion Reactions

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A hemolytic transfusion reaction (HTR) is the accelerated clearance or lysis of transfused red cells because of immunologic incompatibility. It is distinguished from autoimmune hemolysis or nonimmune causes of shortened survival of transfused red cells. HTR may occur when antigen-positive red cells are transfused to a patient with a preexisting alloantibody, or when a recently transfused patient makes a new alloantibody.

The great majority of HTRs are a result of Red Blood Cell (RBC) transfusion. However, HTRs may also result from transfusion of plasma-containing blood components, such as Fresh Frozen Plasma or Platelets, which contain red cell antibodies but very few, if any, red cells.<sup>1,2</sup> Occasionally, HTRs may be caused by incompatibility between red cells from one donor and antibody-containing plasma from a different donor transfused to the same recipient.<sup>3</sup>

Incidence

The actual incidence of HTR is difficult to determine. Much of the data are derived from retrospective studies and are likely to suffer from underreporting. A prospective study of bedside transfusion errors over a 15-month period found that major errors occurred in 1.24% of transfused patients.<sup>4</sup> Although only one of these errors resulted in a clinical reaction, none were reported—indicating that most errors in transfusion practice are not recognized at the time. In some cases, underlying medical conditions such as liver disease, sickle cell anemia, or bleeding may make a definite diagnosis difficult, particularly in delayed HTR (DHTR).

The reported incidence of HTR depends to some degree on the recipient patient population, and is likely to be higher in academic medical centers with heavily transfused patient populations. Reports from blood centers have largely relied on surveys of transfusion services, which may result in underestimation. Improvement in antibody identification techniques over time,

as well as increased knowledge of immunohematology, have undoubtedly resulted in a progressive decrease in HTRs.

Estimates of the incidence of HTR derived from both blood centers and transfusion services are summarized in Table 52-1. Some reports do not differentiate between acute and delayed reactions, or between intravascular and extravascular hemolysis. HTRs have not necessarily been differentiated from serologic reactions without hemolysis. Overall, there has been an apparent increase in the incidence of DHTR and a decreased in acute HTR (AHTR) with time. Factors contributing to a higher rate of delayed reactions include improved serologic detection, longer survival of transfusion patients, and increasing total number of red cell transfusions. Because most patients receive more than one RBC unit, estimates of the incidence of HTR per transfused patient range from 1:854 to 1:524, which is higher than the incidence per unit transfused.<sup>11,15</sup> The population incidence rate of DHTR has been estimated at 1.69 events per 100,000 population per year.<sup>15</sup>

Table 52-1. Estimated Incidence of Hemolytic Transfusion Reactions (HTRs)

| Setting                                                             | Period    | HTR Risk Estimate per Unit Transfused |
|---------------------------------------------------------------------|-----------|---------------------------------------|
| <b>Overall HTR (acute and delayed reactions not differentiated)</b> |           |                                       |
| Blood center <sup>5</sup>                                           | 1981-1987 | 1:15,605                              |
| Oncology patients <sup>6</sup>                                      | 1974-1981 | 1:35,739                              |
| <b>Acute HTR</b>                                                    |           |                                       |
| Tertiary care medical center <sup>7</sup>                           | 1964-1973 | 1:12,100                              |
| Tertiary care medical center <sup>8</sup>                           | 1974-1977 | 1:21,222                              |
| Reported transfusion errors <sup>*9</sup>                           | 1990-1999 | 1:77,000                              |
| <b>Delayed HTR</b>                                                  |           |                                       |
| Tertiary care medical center <sup>10</sup>                          | 1964-1973 | 1:11,652                              |
| Tertiary care medical center <sup>18</sup>                          | 1974-1977 | 1:4,015                               |
| Tertiary care medical center <sup>11</sup>                          | 1980-1992 | 1:5,405                               |
| Blood center <sup>12</sup>                                          | 1980-1981 | 1:6,875                               |
| Tertiary care medical center <sup>13</sup>                          | 1986-1987 | 1:9,094                               |
| Tertiary care medical center <sup>14</sup>                          | 1974-1978 | 1:2,339                               |

\*Clinical reactions in ABO transfusion errors.  
<sup>†</sup>Same institution as previous, improved serologic detection.

Rossi's Principles of Transfusion Medicine, 4th Edition, Edited by Toby L. Simon, E. L. Snyder, B. G. Solheim, Christopher, P. Stowell, Ronald, G. Strauss and Marian Petrides © 2009 Blackwell Publishing Ltd. ISBN: 978-1-405-17588-3

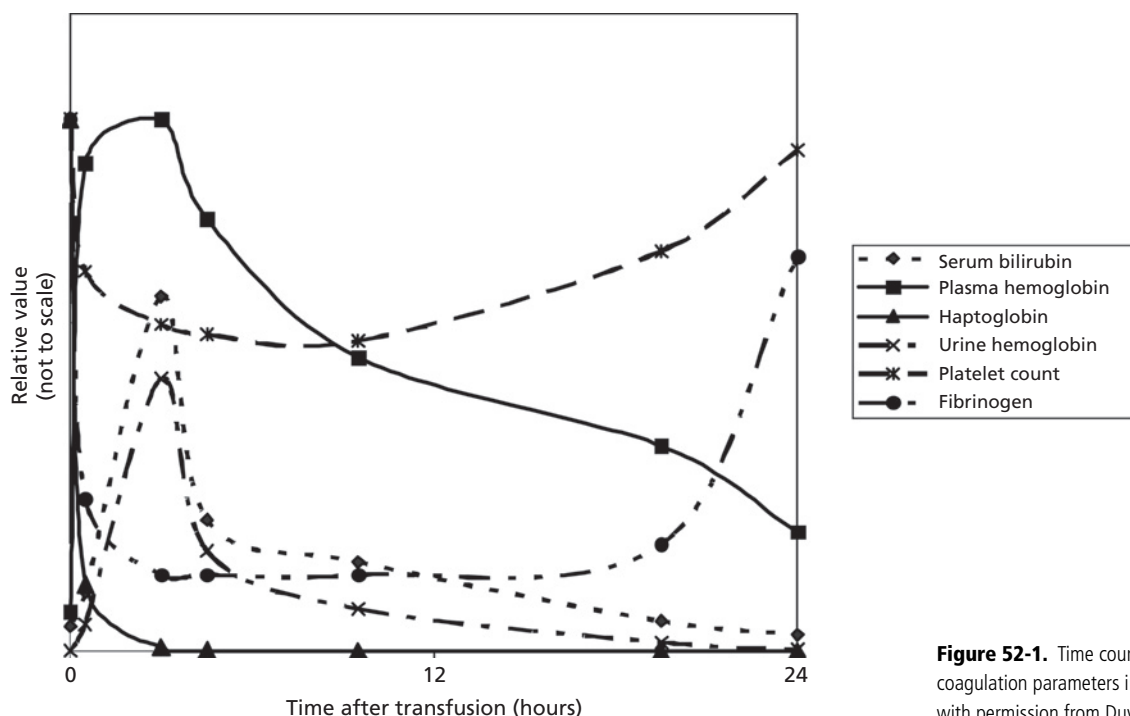


**Figure 52-1.** Time course of hemolytic and coagulation parameters in intravascular HTR. Adapted with permission from Duvall et al.<sup>18</sup>

Since 1996, the Serious Hazards of Transfusion (SHOT) program in the United Kingdom has reported and tracked HTRs. In 2004, HTRs caused by ABO-incompatible RBC transfusions were reported in 19 of 2,607,410 units issued, for a rate of 1:137,000.<sup>16</sup> The number of patients transfused or units transfused was not reported. However, the wastage rate was estimated to be less than 5%. The number of ABO-incompatible transfusions reported annually to SHOT officials since the program's inception has varied from 13 to 36. The number of units issued was not reported for years prior to 2004.

Data on HTRs from the Quebec Hemovigilance System covering 2000 to 2004 have been reported.<sup>17</sup> Over a 5-year period, 47 ABO-incompatible transfusions, 55 AHTRs, and 91 DHTRs out of 7059 total transfusion reactions occurred. The incidence per RBC unit issued was 1:27,318 for ABO incompatibility, 1:14,901 for AHTR, and 1:9313 for DHTR. The most common cause of AHTR was ABO incompatibility, accounting for 30% of reactions.

HTRs are classified as acute or delayed reactions, based on whether they occur within 24 hours or after 24 hours of the implicated transfusion. A more important distinction is between intravascular and extravascular hemolytic reactions. Intravascular hemolysis is characterized by hemoglobinemia and hemoglobinuria. In contrast, extravascular hemolysis lacks these dramatic signs, but is characterized by shortened survival of transfused red cells along with the accumulation of hemoglobin breakdown products. Generally, intravascular hemolysis is seen with AHTR, while extravascular hemolysis is usually seen in DHTR. This distinction is not absolute, however. Occasional acute reactions

result in extravascular hemolysis, and some intravascular hemolytic reactions are delayed.

Accelerated clearance of incompatible red cells is an essential feature of HTRs. Transfusion may also stimulate the production of alloantibody without hemolysis. This phenomenon has been termed delayed serologic transfusion reaction (DSTR), and needs to be differentiated from delayed HTR.<sup>11,13</sup> DSTR is clinically benign, but predisposes to later HTR. Earlier reports of hemolytic reactions have not necessarily differentiated clearly between serologic and true hemolytic reactions.

## Signs and Symptoms

There is a broad range of initial clinical presentations of HTR. The typical progression of intravascular and extravascular hemolysis is shown in Fig 52-1 and Fig 52-2. In all cases, there is an unexpected degree of anemia from the loss of transfused red blood cells. In some cases, particularly with extravascular hemolysis, this is the only clue to HTR. Some reactions, both immediate and delayed, are asymptomatic. In intravascular hemolysis, however, most symptomatic patients experience fever and/or chills. Nausea or vomiting, pain, dyspnea, and hypotension or tachycardia are also common initial symptoms. The reported pain may localize to the infusion site, back, flanks, chest, groin, or head. The cause of pain in HTR is unclear, but is most likely the result of direct stimulation of nociceptive nerves in perivascular



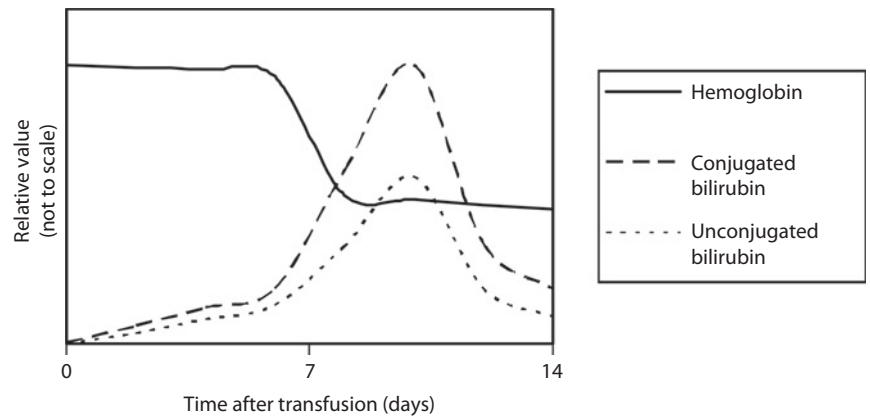


**Figure 52-2.** Time course of hemolytic parameters in extravascular hemolysis. Adapted from Cummins et al.<sup>19</sup>

tissue by bradykinin generated from activation of the complement system.<sup>20</sup>

In extravascular hemolysis, fever and/or chills are the most commonly reported initial symptoms. Jaundice also may be an initial sign because elevation of serum bilirubin occurs with both intravascular and extravascular hemolysis. The degree of hyperbilirubinemia depends upon the patient's liver function and rate of red cell destruction. Conjugated and unconjugated bilirubin fractions tend to follow a parallel course, peaking at the same time.<sup>19</sup> Delta bilirubin, a minor albumin-bound fraction, persists past the peak of total bilirubin, and is a useful clue to previous hemolysis.

Although HTR typically presents during or shortly after the offending transfusion, the time from transfusion to clinical presentation of DHTR is quite variable. Most delayed reactions present within 2 weeks after transfusion, but the initial presentation may be up to 6 weeks later, because of the time required for antibody production.

## Complications

Autoantibody is found in about 28% of patients concomitantly with alloantibody, although the reported range is 15% to 53%.<sup>21</sup> A positive direct antiglobulin test (DAT) caused by IgG may persist for many months, and there may be evidence of complement deposition on autologous red cells, with a positive DAT caused by C3 persisting for up to 100 days.<sup>22</sup> In most cases, there is no evidence of loss of autologous red cells; however, so-called "bystander hemolysis" can occur. It is not possible to distinguish between autoimmune hemolytic anemia (AIHA) and bystander hemolysis by serologic testing alone.

Patients with sickle cell anemia can present a particular challenge. HTR can precipitate sickle crisis. Factors that contribute to the occurrence of sickle crisis include increased oxygen consumption resulting from fever, the relative loss of circulating hemoglobin A compared to hemoglobin S, and the release of vasoactive mediators causing reduction of local blood flow. HTRs may be particularly

severe in patients with sickle cell disease. In such reactions the degree of anemia may actually be greater than before transfusion, probably because of bystander hemolysis of autologous red cells. This phenomenon has been termed the sickle cell HTR syndrome.<sup>23</sup> In addition to hemolysis, there is often suppression of erythropoiesis as indicated by a marked drop in the reticulocyte count. Demonstration of an increase in corrected reticulocyte count, increase in the absolute number of hemoglobin-S-containing red cells, or decrease in the ratio of red cell hemoglobin to reticulocyte hemoglobin can indicate the occurrence of hyperhemolysis.<sup>23-25</sup> Pain crisis in a sickle cell patient following transfusion should suggest the occurrence of sickle cell HTR syndrome. Further transfusion in this setting may exacerbate the anemia and even result in fatality. Serologic studies often do not provide an explanation for HTR in these patients, because the causative antibody can be at an undetectable level in the serum during the reaction. In addition, the presence of multiple alloantibodies may make the serologic diagnosis difficult.

Hypotension occurs in some cases of intravascular HTR but is rare in extravascular reactions. Complement activation is likely to be the most important determining factor. The anaphylatoxins C3a, C4a, C5a, and C5a-des-arg are released during immune hemolysis. Additionally, consumption of C1-esterase inhibitor contributes to activation of the kinin pathway, leading to generation of bradykinin.<sup>26</sup> The proinflammatory cytokines tumor necrosis factor (TNF) and interleukin (IL)-1 produced by phagocytes during HTR also may contribute to hypotension and shock.

Impairment of renal function is seen in both intravascular and extravascular HTR, although it is more common in the former. The degree of renal function abnormality varies from an asymptomatic elevation of serum blood urea nitrogen (BUN) and creatinine to complete anuria. Both hypotension and intravascular coagulation contribute to renal impairment. Thrombus formation in renal arterioles caused by disseminated intravascular coagulation (DIC) may cause cortical infarcts. Free hemoglobin contributes to renal injury, causing so-called pigment nephropathy. Experimental evidence indicates that hemoglobin is toxic to renal tubular epithelium cells.<sup>27</sup>

Intravascular hemolysis modulates blood pressure and local blood flow through alterations in the metabolism of the potent physiologic vasodilator nitric oxide (NO). NO can combine with heme and thiol groups of hemoglobin.<sup>28</sup> In oxyhemoglobin, NO causes reduction of ferrous iron ( $\text{Fe}^{2+}$ ) to form ferric methemoglobin [ $\text{Hb}(\text{Fe}^{3+})$ ]. NO combines with deoxyhemoglobin to form  $\text{Hb}(\text{Fe}^{2+})\text{NO}$ , but does not cause reduction. Scavenging of NO by free hemoglobin through these pathways results in vasoconstriction and hypertension. NO can also combine with cysteine on the beta globin chain to form S-nitrosohemoglobin (SNO-Hb). This process is reversible, so SNO-Hb can act as a NO donor with resultant vasodilation. Free hemoglobin reacts with NO much more rapidly than does intraerythrocytic hemoglobin.<sup>29</sup> The effects of intravascular hemolysis may be very similar to the infusion of stroma-free hemoglobin. Indeed, prominent hypertensive effects limited early trials of hemoglobin solutions as a blood substitute.<sup>30</sup>

Disseminated intravascular coagulation occurs in intravascular HTR, but is relatively rare. Rarer still is the occurrence of DIC in extravascular HTR. The production of proinflammatory cytokines is likely to be a major factor in DIC. It can be difficult to distinguish DIC from other causes of coagulopathy, particularly in massive transfusion or liver disease. Uncontrolled bleeding caused by DIC may be the initial manifestation of an acute HTR, particularly in the intraoperative setting. Unfortunately, if HTR is not recognized early in this situation, more incompatible blood may be transfused in an attempt to keep up with blood loss. In one report, nine of 35 patients experiencing HTR while under anesthesia received 4 to 6 additional units because of excessive bleeding.<sup>31</sup>

Fatality from HTR is usually associated with intravascular hemolysis, but severe extravascular reactions may also cause death.<sup>7,32</sup> It is difficult to estimate the actual rate of mortality, but the best available data are derived from reports to regulatory agencies. A review of transfusion-associated deaths reported to the Food and Drug Administration (FDA) between 1976 and 1985 attributed 158 fatalities to acute hemolysis and 26 to delayed hemolysis.<sup>33</sup> Assuming that 100 million RBC units were transfused in this time, the mortality rate for AHTR would be 1:630,000 and that for DHTR would be 1:3,850,000 per unit transfused. Similarly, assuming that 30 million patients were transfused in this time, the per patient mortality rate for AHTR would be 1:190,000, and for DHTR would be 1:1,150,000. An earlier review of transfusion fatalities reported to the FDA covering 1976 to 1978 estimated the death rate from HTR to be 1:587,000 per unit transfused.<sup>34</sup> A recent update has extended the analysis of FDA fatality reports through 1995.<sup>35</sup> Since 1990 the number of deaths reported to the FDA annually has increased, probably because of increased vigilance in reporting. The total number of deaths (151) from acute and delayed hemolysis reported between 1986 and 1995 was essentially unchanged from the prior 10 years. However, acute hemolysis accounted for a higher proportion of deaths (62%) during the first decade, than during the second decade (50%). This difference was caused by a higher incidence

of deaths from other causes including bacterial contamination and acute lung injury in the later report. The total number and proportion of deaths from delayed HTR was unchanged between the two reporting periods (26 or 10% vs 29 or 10%).

An analysis of blood transfusion errors reported to the New York State Health Department, from January 1990 to October 1991, reported a death rate of 1:600,000 per unit transfused.<sup>36</sup> When these data were extended to the period 1990 to 1999, the fatality rate from erroneous administration of RBC units was 1:1,800,000.<sup>9</sup> It is not surprising that different studies result in discrepant estimates of mortality rate, because these figures are based on small numbers of events.

The cumulative SHOT data from 1996 to 2004 includes 245 ABO-incompatible transfusions. Among these, death related to transfusion occurred in 7% of cases, major morbidity in 22%, and no ill effect in 63%.<sup>16,37</sup>

Mortality is dependent on the amount of incompatible red cells transfused. A review of 41 HTRs causing acute renal failure indicated that no deaths occurred among patients receiving less than 500 mL of incompatible blood; there was 25% mortality in the group receiving 500 to 1000 mL and 44% mortality among those receiving greater than 1000 mL of incompatible blood.<sup>31</sup> However, transfusion of even small amounts of incompatible blood is not necessarily safe. At least 12 deaths have been reported to the FDA involving transfusion of less than 1 unit of blood.<sup>33</sup>

## Causes of Hemolytic Transfusion Reactions

Hemolytic transfusion reactions usually result from inadvertent administration of incompatible blood components, or the failure to detect a potential incompatibility. Rarely, incompatible blood components are deliberately transfused before ABO-incompatible marrow infusion or when compatible blood cannot be obtained, with an expectation of possible HTR. Among transfusion-related fatalities from acute hemolysis reported to the FDA during 1976 to 1985, 86% were the result of process errors.<sup>33</sup> Among these errors, 10% occurred in phlebotomy and ordering departments, 33% occurred within the blood bank, and 57% occurred during transfusion administration.

These frequencies are similar to reported transfusion errors in New York State, where 22% occurred in phlebotomy and ordering departments, 32% occurred within the blood bank, and 46% occurred during transfusion administration.<sup>36</sup> More recent figures indicate that non-blood-bank errors account for 56%, blood bank errors account for 29%, and compound errors account for the remaining 15% of transfusion errors.<sup>9</sup> In part, differences between the FDA and New York figures are accounted for by the inclusion of nonfatal errors in the latter. Serendipitously, a transfusion error may result in administration of compatible blood components, as occurred in a third of the reported New York State events.

Misidentification of a pretransfusion sample has been termed “wrong blood in tube” (WBIT). A study covering 62 institutions

in 10 countries performed by the Biomedical Excellence for Safer Transfusion Working Party of the International Society for Blood Transfusion has assessed the frequency of WBIT.<sup>38</sup> On the basis of results from over 690,000 samples, it was determined mislabeling occurred in 1 in every 165 samples. Two countries with national patient identification systems, Sweden and Finland, had miscollection rates too low to quantify. Outside these nations, miscollected samples demonstrating WBIT occurred at a median rate of 1 in every 1986 samples. The apparent discrepancy between mislabeling and WBIT is because not all mislabeled tubes are miscollected.

Data on the failure of pretransfusion tests to detect potential incompatibility are scant. This is more likely to be a factor in DHTR than in AHTR. A Mayo Clinic study suggested that at least five of 37 cases (14%) of delayed HTR could have been prevented by improving the sensitivity of red cell antibody screening procedures.<sup>8</sup>

The deliberate, physician-guided administration of incompatible blood components may occur in platelet transfusion, urgent transfusion required in patients with multiple alloantibodies or antibodies to high-incidence antigens, or in marrow transplantation. ABO-incompatible red cells have been successfully administered to patients before major ABO-incompatible marrow transplants in an effort to reduce the titer of isoagglutinins.<sup>39</sup> These patients have predictable, but manageable, reactions. In a retrospective study of 35 patients receiving major ABO-incompatible peripheral blood hematopoietic progenitor cell transplants who were transfused with 1 ABO-incompatible RBC unit, 23 had no clinical reaction, and four had severe reactions necessitating discontinuation of the transfusion.<sup>40</sup> Some patients who have received incompatible transfusions involving non-ABO antibodies were treated with high-dose intravenous immunoglobulin (IVIG) before transfusion and have not experienced HTR.<sup>41</sup>

High levels of anti-A or anti-B in platelet concentrates, particularly apheresis products, can cause acute hemolysis.<sup>42-45</sup> There is considerable variability in the strength of the implicated antibody, but a high-titer isoagglutinin is usually present in donor plasma when hemolysis occurs. A study of apheresis group O platelets found that 26% had anti-A or anti-A,B titer of 64 or greater, a level that has been associated with HTR.<sup>46</sup> A study of pooled whole-blood-derived platelets found 60% of group O pools had anti-A titer at least 64.<sup>47</sup> Repeated platelet transfusions within a brief period can result in sufficient accumulation of anti-A to cause severe acute hemolysis.<sup>48</sup> However, there is no clear consensus as to what constitutes a critical titer of ABO antibodies. At variance with reports of acute hemolysis, apheresis platelet concentrates are often transfused across ABO groups without adverse consequences. In one study 16 patients who received both ABO-compatible and -incompatible platelet concentrates were evaluated for evidence of hemolysis.<sup>49</sup> There was no difference between pre- and posttransfusion hemoglobin levels in 24 paired transfusion episodes.

## Diagnosis

Diagnosis of HTR requires clinical suspicion, especially when the transfusion occurred days to weeks previously. The initial laboratory evaluation includes confirmation of the ABO group, a test for free hemoglobin, and a DAT performed on a postreaction blood specimen (Table 52-2). Visual inspection of the postreaction plasma can detect hemoglobin in the range of 20 to 50 mg/dL, equivalent to the lysis of approximately 10 mL of red cells in an adult.<sup>50</sup> It should be remembered that free serum hemoglobin also may be present in nonimmune hemolysis, red cell fragility syndromes, hemoglobinopathies, severe burns, polyagglutination, or infusion of hemoglobin-based oxygen-carrying solutions. A common cause of a false-positive test result for free hemoglobin is drawing a sample through an indwelling catheter using inappropriate technique. Percutaneous intravascular thromboectomy can cause marked hemoglobinemia.<sup>51</sup> A false-negative test result for free hemoglobin may occur if too much time has been allowed to elapse before obtaining the postreaction specimen.

**Table 52-2.** Laboratory Investigation of Hemolytic Transfusion Reactions

### First-tier investigation

- Posttransfusion serum hemoglobin (qualitative)
- Posttransfusion direct antiglobulin test
- Confirmation of posttransfusion ABO/Rh

### Second-tier investigation

- Repeat pretransfusion ABO/Rh
- Pre- and posttransfusion antibody screen
- Repeat special antigen typing
- Crossmatch with pre- and postreaction specimens

### Third-tier investigation

- Antibody identification panels on pre- and postreaction samples
- Enhanced antibody screening method: PEG, extended incubation, gel, enzymes
- Red cell eluate on pre- and postreaction samples
- Investigation of transfusion technique and blood storage conditions
- Check of the blood bag, tubing, and segments for hemolysis
- Enhanced crossmatches: PEG, enzymes
- Minor crossmatches of implicated units
- Antibody detection tests on donor units
- Tests for polyagglutination
- Hemoglobin electrophoresis
- Quantitative serum hemoglobin
- Serum haptoglobin
- Serum bilirubin (conjugated and unconjugated)
- Urine hemoglobin and hemosiderin
- Bacterial culture and Gram's stain of blood bags
- Serum BUN and creatinine
- Peripheral blood smear
- Serial hemoglobin, hematocrit, and platelet count
- Blood coagulation studies (PT, aPTT, fibrinogen, FDP)
- DAT on donor units

PEG = polyethylene glycol; BUN = blood urea nitrogen; PT = prothrombin time; aPTT = activated partial thromboplastin time; FDP = fibrin degradation product; DAT = direct antiglobulin test.

A low level of free hemoglobin may be difficult to detect in an icteric specimen.

The DAT may be positive because of the effects of drugs, autoimmune disease, or autoantibodies. If the postreaction DAT is positive, then a test should be performed on a stored pre-reaction sample. If the pre-reaction sample is also a positive, then the test is not valid for the purpose of detecting, or excluding, the presence of alloantibody-coated transfused cells and further testing is required. The DAT will be negative if transfused antigen-positive cells have been cleared from circulation. In addition, when small numbers of antibody-coated allogeneic red cells are present, a DAT performed by routine methods may not be sufficiently sensitive. In a study that compared the relative sensitivity of DAT performed with monospecific IgG antiglobulin technique, flow cytometry, and antibody elution, the DAT could detect 10% antibody-coated cells.<sup>52</sup> Antibody could be detected in the eluate of the samples with as little as 1% antibody-coated red cells present, although in some cases it was not nearly as sensitive. Flow cytometry, however, was consistently the most sensitive method with a detection limit of approximately 1%.

The hemoglobin and antiglobulin tests are useful for screening purposes. However, if either of these is positive, or if there is a strong clinical suspicion, then further testing must be performed. At this point, the implicated blood bags with their attached administration sets should be returned to the blood bank, and all units dispensed to the patient should be returned and quarantined. Units that were transfused within the past 24 hours should also be identified. The ABO grouping and Rh typing of both pre- and postreaction blood specimens should be repeated. Care should be taken to look for mixed-field agglutination. The antibody screen should be repeated on both specimens, and any special antigen typing of donor units should be repeated.

Blood from saved segments or recovered bags and tubing of units transfused within 24 hours should be crossmatched against both the pre- and postreaction specimens. This testing should include a 37°C reading and an indirect antiglobulin test. If a large number of units have been transfused in the last 24 hours, the blood bank medical director may elect to substitute reconfirmation of ABO grouping of donor units. Negative results in these investigations usually rule out HTR, except in unusual circumstances.

Other causes of hemolysis or shortened red cell survival that should be considered in the differential diagnosis of HTR are listed in Table 52-3. Nonimmune hemolysis can have a similar clinical presentation to HTR. Lysis of red cells can be caused by overheating in a blood warmer, or by freezing. Hemolysis can also be caused by inadequate removal of glycerol from frozen red cells or by attempting to force blood through a filter or small-bore needle. The transfusion of outdated blood has been reported to cause hemoglobinuria and transient hemodynamic, pulmonary, and renal changes.<sup>53</sup> Transfusion administered with hypotonic solutions or some drugs may also cause hemolysis.<sup>54</sup> Intravenous dimethylsulfoxide infusion has been reported to mimic HTR.<sup>55</sup> In general, patients tolerate the infusion of

hemolyzed blood remarkable well. Often the only sign of an adverse event is hemoglobinuria. However, deaths caused by the transfusion of hemolyzed blood have been reported.<sup>33</sup>

Some hematologic abnormalities, particularly autoimmune hemolytic anemia, can have presentations similar to HTR. Patients with congenital hemolytic anemias, such as glucose-6-phosphate dehydrogenase deficiency, may manifest hemolysis after blood transfusion.<sup>56</sup> Conversely, blood donated by individuals with glucose-6-phosphate dehydrogenase deficiency can cause hemoglobinemia and hyperbilirubinemia in transfusion recipients.<sup>57</sup>

Establishing the diagnosis of HTR may be particularly difficult in patients with liver disease, autoimmune hemolytic anemia, sickle cell anemia, or active bleeding. In chronic liver disease there is often a positive DAT, hyperbilirubinemia, and elevated lactate dehydrogenase (LDH). The clinical and laboratory presentation of AIHA may be identical to HTR. Concern has been raised that transfusion may aggravate hemolysis in

**Table 52-3.** Differential Diagnosis of Hemolytic Transfusion Reactions

- 
- Alloantibody-induced hemolysis
  - Delayed serologic transfusion reaction
  - Autoimmune hemolytic anemia
  - Cold hemagglutinin disease
  - Nonimmune hemolysis
    - Incompatible fluids
    - Improper storage
    - Malfunctioning blood warmer
    - Small needles, high hematocrit
    - Improper deglycerolization
    - Infusion pumps
    - Bacterial contamination
    - Mechanical thrombectomy
  - Hemolytic anemia
    - G6PD deficiency
    - Congenital spherocytic anemia
  - Hemoglobinopathies
    - Sickle cell disease
    - Sickle cell transfusion reaction syndrome
  - Drug-induced hemolysis
  - Microangiopathic hemolytic anemias
    - Thrombotic thrombocytopenic purpura
    - Hemolytic uremic syndrome
    - HELLP syndrome
  - Bleeding
  - Artificial heart valve dysfunction
  - Paroxysmal nocturnal hemoglobinuria
  - Polyagglutination
  - Infections
    - *Clostridium perfringens*
    - Malaria
    - Babesiosis
- 

G6PD = glucose-6-phosphate dehydrogenase; HELLP = hemolysis, elevated liver enzymes, and low platelet count.

AIHA, although one published study has suggested that this is not usually the case even in the face of serologic incompatibility.<sup>58</sup> Characteristically, in both bleeding and AIHA there is proportionate loss of both autologous and transfused red cells. One indication of HTR in these settings is the persistence of transfused red cells that lack the implicated antigen, but the absence of transfused cells bearing the antigen.

Resorption of a hematoma can have manifestations very similar to extravascular HTR. Such patients have an unconjugated hyperbilirubinemia, elevated LDH, and depressed haptoglobin levels. In addition, the presence in the serum of fibrin degradation products (FDPs) from the hematoma may be confused with DIC. In these patients, as in bleeding patients, persistent circulating antigen-positive red cells and a negative posttransfusion DAT are evidence against the diagnosis of HTR.

The serologic specificity of a red cell antibody is an indication of its clinical significance. However, there is not an absolute correlation between specificity and presence or absence of red cell destruction. The general clinical significance of many red cell antibody specificities is summarized in Table 52-4.

## Pathophysiology

The pathophysiologic mechanisms involved in HTR are not well understood. There are essentially three phases: antibody-antigen interaction, phagocytosis and inflammatory cell activation, and systemic response. Initially, there is a binding of antibody to red cell antigens, which can result in complement activation. Second, immunoglobulin- and complement-coated cells interact with phagocytes, resulting in clearance of red cells and activation of phagocytes. Third, the inflammatory mediators produced in the first two phases act on a variety of cell types, causing a clinical manifestations of HTR.

The course of immune hemolysis is determined by antigen site density, immunoglobulin class of the alloantibody, and activation of complement. ABO antigens are present in high numbers on a red cell surface, approximately  $5 \times 10^5$  per cell.<sup>50</sup> In contrast, there are  $10^3$  to  $10^4$  antigens per cell in the Rh, Kell, Kidd, and Duffy systems. Complement fixation is facilitated by close proximity of antigens that allows bridging of IgG molecules by C1q. However, IgM antibodies can fix complement without a requirement for bridging between molecules. IgM antibodies are common in the ABO system, but relatively unusual as alloantibodies to other antigens. Activation of the classical pathway of complement proceeds from C1q binding through C3 activation. Cleavage of C3 results in C3a liberation into circulation and C3b deposition on the red cell membrane. Activated C3 may then cleave C5 with release of C5a. Assembly of the membrane attack complex then may proceed with resultant intravascular hemolysis. Factor I, also known as C3b inactivator, is the major regulator of C3b activity. Cleavage of membrane-bound C3b by factor I results in the generation of iC3b and release of the small peptide fragment C3c. This terminates the complement cascade

because iC3b is enzymatically inactive. iC3b is further degraded into C3dg and C3d by factor I and trypsin-like proteinases.

Erythrophagocytosis results from interaction of immunoglobulin- and/or complement-coated red cells with phagocyte receptors. Cell-bound antibodies promote red cell clearance primarily through interaction of the Fc portion of IgG with specific receptors. Among the IgG receptors, Fc $\gamma$ RI principally mediates red cell phagocytosis by monocytes.<sup>59</sup> However, this receptor has a high affinity for monomeric IgG, and is blocked by normal serum concentrations of IgG.<sup>60</sup> Fc $\gamma$ RIII appears to be the most important IgG receptor on splenic macrophages in alloimmune and autoimmune red cell clearance, as well as in autoimmune thrombocytopenia.<sup>61-63</sup> The principal complement receptor expressed by macrophages and monocytes, CR3, primarily recognizes iC3b. Receptors for C3a and C5a are present on a wide variety of cells including monocytes, macrophages, neutrophils, platelets, endothelium, and smooth muscle. The physiologic

**Table 52-4.** General Clinical Significance of Red Cell Antibodies

| Blood Group System       | Generally Clinically Significant* Specificities        | Generally Clinically Insignificant Specificities       |
|--------------------------|--------------------------------------------------------|--------------------------------------------------------|
| ABO, H                   | All                                                    | -A1 not reactive at 37°C                               |
| Lewis                    | -Le <sup>a</sup> , -Le <sup>a</sup> +Le <sup>b</sup>   | -Le <sup>b</sup>                                       |
| P                        | -P, -P+P1+Pk (Tj <sup>a</sup> )                        | -P1, -P <sup>k</sup>                                   |
| I/i                      | -I, -i                                                 | -IH, -IA, -IB, -iH, -IP1 not reactive at 37°C          |
| Rh                       | All                                                    |                                                        |
| Duffy                    | All                                                    | Possibly -Fy6                                          |
| MNSs                     | All                                                    | -M not reactive at 37°C                                |
| Lutheran                 | -Lu <sup>b</sup>                                       | -Lu <sup>a</sup>                                       |
| Kell                     | All                                                    |                                                        |
| Kidd                     | All                                                    |                                                        |
| Cartwright               | -Yt <sup>a</sup> , -Yt <sup>b</sup>                    |                                                        |
| Diego                    | -Di <sup>a</sup> , -Dj <sup>b</sup> , -Wr <sup>a</sup> |                                                        |
| Colton                   | -Co <sup>a</sup> , -Co3                                | -Co <sup>b</sup>                                       |
| Dombrock                 | -Do <sup>a</sup> , -Do <sup>b</sup>                    |                                                        |
| Cromer                   | -Cr <sup>a</sup> , -Tc <sup>a</sup>                    |                                                        |
| Augustine                | -At <sup>a</sup>                                       |                                                        |
| Vel                      | -Vel                                                   |                                                        |
| Lan                      | -Lan                                                   |                                                        |
| Sid                      |                                                        | -Sd <sup>a</sup>                                       |
| LW                       |                                                        | All                                                    |
| Gerbich                  |                                                        | All                                                    |
| Xg                       |                                                        | All                                                    |
| Scianna                  |                                                        | All                                                    |
| Chido/Rogers             |                                                        | All                                                    |
| Indian                   | -In <sup>b</sup>                                       | -In <sup>a</sup>                                       |
| Cost/York                |                                                        | -Cs <sup>a</sup> , -Yk <sup>a</sup> , -Yk <sup>b</sup> |
| Knops/McCoy              |                                                        | -Kn <sup>a</sup> , -Kn <sup>b</sup>                    |
| JMH                      |                                                        | -JMH                                                   |
| Holly/Gregory (Dombrock) |                                                        | -Gy <sup>a</sup> , -Hy                                 |
| Bg (HLA)                 |                                                        | All                                                    |

\*Resulting in hemolytic transfusion reaction or decreased red cell survival.

effects of C3a and C5a include oxygen radical production, granule enzyme release, leukotriene production, NO production, and cytokine production. These low-molecular-weight peptides can also produce vasodilation and bronchoconstriction.

Ligation of phagocyte receptors results in cellular activation and production of inflammatory response factors. An experimental model of ABO incompatibility has suggested that monocytes are the leukocyte subpopulation most directly involved in AHTR.<sup>64</sup> Incompatible red cells induce a reduction in CD14 and increase in CD44 expression on monocytes in whole blood. After 24 hours incubation with incompatible red cells, monocytes showed particularly high levels of CD44. These data demonstrate that monocyte activation is critical in the development of intravascular HTR.

Immune hemolysis stimulates the production of a variety of cytokines that are crucial to the initiation, maintenance, and ultimate resolution of HTR (Table 52-5). ABO incompatibility with intravascular HTR strongly stimulates production of TNF- $\alpha$  and the chemokines CXCL8 (IL-8) and CCL2 (MCP-1).<sup>65-68</sup> TNF- $\alpha$  is an early response, appearing in plasma within 2 hours, and has potent proinflammatory effects, including pyrogenic activity, leukocyte activation, stimulation of procoagulant activity, and expression of a large number of gene products related to the inflammatory response. TNF- $\alpha$  produced in blood during ABO incompatibility will stimulate endothelial cells to express leukocyte adhesion molecules, chemotactic cytokines, and

procoagulant activity.<sup>69</sup> CXCL8 and CCL2 produced in blood during ABO incompatibility appear later than TNF- $\alpha$  and reach very high levels. CXCL8 primarily activates neutrophils to undergo the respiratory burst, release granule contents and alter surface adhesion molecules.<sup>70</sup> CCL2 is primarily a chemotactic and activating factor for monocytes.<sup>71</sup>

There is also evidence for the production of cytokines in IgG-mediated extravascular hemolysis.<sup>72,73</sup> There appear to be two categories of cytokine responses in this setting: those produced at high levels (greater than 1 ng/mL by 24 hours), and others produced at lower level (in the range of 100 pg/mL).<sup>72</sup> Low-level cytokine responses include IL-1 $\beta$ , IL-6, and TNF- $\alpha$ . CXCL8 is a high-level response with a time course similar to that of ABO incompatibility. In contrast to the setting of ABO incompatibility, TNF- $\alpha$  is produced in a delayed fashion in response to IgG-coated red cells, achieving a level of less than 100 pg/mL. However, cell-associated TNF- $\alpha$  can be demonstrated by immunocytochemical staining in monocytes engaged in erythrophagocytosis.

While the in-vitro models employed in these studies are not directly comparable, these findings do suggest a possible reason for the clinical differences between intravascular and extravascular HTRs. In the former case, TNF- $\alpha$  is released into systemic circulation where it can have diverse effects on many cell types; in the latter case, TNF- $\alpha$  effects may be confined to the site of erythrophagocytosis, primarily the spleen. Both IL-1 $\beta$  and IL-6 produced by monocytes in response to IgG-coated red cells increase progressively over 24 hours to levels approximating 100 pg/mL. Because IL-1 $\beta$  and IL-6 are B-cell growth and differentiation factors, the production of these two cytokines promotes the production of red cell allo- and autoantibodies that are often associated with DHTRs.

IgG-mediated hemolysis also results in the production of the IL-1 $\beta$  inhibitor IL-1ra.<sup>74</sup> Significant levels of IL-1ra appear in a parallel fashion to IL-1 $\beta$ . Immunocytochemical staining has demonstrated strong reactivity for IL-1ra in monocytes engaged in erythrophagocytosis. Northern blot analysis of mononuclear cell RNA shows that IL-1 $\beta$  gene expression precedes that of IL-1ra in response to IgG-coated red cells. However, neutralizing antibodies to IL-1 $\beta$  do not suppress either IL-1ra or IL-1 $\beta$  gene expression in this setting. Therefore, it appears that IL-1ra production is a primary response to the IgG-coated red cell stimulus, rather than an autocrine phenomenon induced by initial IL-1 $\beta$  production. Treatment of mononuclear cells with the steroid dexamethasone inhibits IL-1ra production in response to IgG-coated red cells. These data suggest the possibility that the clinical variability of DHTR, and some of the clinical differences from intravascular HTR, may be accounted for, in part, by the relative balance of IL-1 $\beta$  and IL-1ra production.

Labile blood pressure is a feature of severe HTR, particularly with intravascular hemolysis. Both complement activation products such as C5a and cytokines such as IL-1 $\beta$  and TNF- $\alpha$  can contribute to hypotension. The common pathway of these mediators is the production of NO by endothelial cells. NO, in

Table 52-5. Cytokines Involved in Immune Hemolysis

| Terminology                                                                                                                                                                                                                                                   | Biologic Activities                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <ul style="list-style-type: none"><li>• Proinflammatory cytokines:<ul style="list-style-type: none"><li>– Interleukin-1 (IL-1<math>\beta</math>)</li><li>– Tumor necrosis factor (TNF-<math>\alpha</math>)</li></ul></li><li>– Interleukin-6 (IL-6)</li></ul> | <ul style="list-style-type: none"><li>• Fever</li><li>• Hypotension, shock, death (synergy)</li><li>• Mobilization of leukocytes from marrow</li><li>• Activation of T cells and B cells</li><li>• Induction of cytokines (IL-1<math>\beta</math>, IL-6, CXCL8, TNF-<math>\alpha</math>, CCL5)</li><li>• Induction of adhesion molecules</li><li>• Induction of procoagulants</li></ul>                                                                                         |
| <ul style="list-style-type: none"><li>• Chemokines:<ul style="list-style-type: none"><li>– CXCL8</li><li>– CCL5</li></ul></li></ul>                                                                                                                           | <ul style="list-style-type: none"><li>• Fever</li><li>• Acute phase protein response</li><li>• B-cell antibody production</li><li>• T-cell activation</li><li>• Chemotaxis of neutrophils</li><li>• Chemotaxis of lymphocytes</li><li>• Neutrophil activation</li><li>• Basophil histamine release</li><li>• Chemotaxis of monocytes</li><li>• Induction of respiratory burst</li><li>• Induction of adhesion molecules</li><li>• Induction of IL-1<math>\beta</math></li></ul> |
| <ul style="list-style-type: none"><li>• Anti-inflammatory cytokines:<ul style="list-style-type: none"><li>– Interleukin-1 receptor antagonist (IL-1ra, IRAP)</li></ul></li></ul>                                                                              | <ul style="list-style-type: none"><li>• Competitive inhibition of IL-1 type I and II receptors</li></ul>                                                                                                                                                                                                                                                                                                                                                                        |

turn, causes relaxation of vascular smooth muscle. Hypotension and deposition of thrombi in arterioles, which impair cortical blood flow, are the major factors that contribute to renal failure. In addition, there may be direct effects of inflammatory mediators on the kidneys.

There are several mechanisms by which HTR results in intravascular coagulation. TNF- $\alpha$  produced during immune hemolysis can induce tissue factor expression by endothelial cells.<sup>69</sup> Tissue factor is an initiator of the extrinsic pathway that functions as a cofactor for Factors VII and VIIa to accelerate the activation of Factors IX and X. TNF- $\alpha$  and IL-1 $\beta$ , acting on endothelial cells, will also decrease the cell surface expression of thrombomodulin. Thrombomodulin is normally present on endothelial cells, and binds thrombin to activate the coagulation inhibitor protein C. Intravascular hemolysis, as in ABO incompatibility, will also induce procoagulant activity in blood leukocytes, largely because of tissue factor expression.<sup>74</sup> This cellular procoagulant is partly inhibited by blocking antibodies to tissue factor and partly dependent of the multifunctional adhesion protein CD11b.

Knowledge of the pathophysiology of HTR has been limited by the lack of good animal models. However, a recently developed model using transgenic mice expressing human glyophorin A holds promise.<sup>75</sup> Administration of IgM and IgG anti-GPA results in intravascular or extravascular hemolysis, respectively. Dependence on active complement and Fc $\gamma$  receptors has been shown.

## Therapy

Patients who have minimal symptoms are best managed by careful observation. However, early vigorous intervention in severe reactions saves lives. Therapeutic options in HTR are

summarized in Table 52-6. The severity of HTR is directly related to volume and rate of infusion of incompatible blood. Thus, early recognition, stopping transfusion, and preventing the transfusion of additional incompatible units is the first essential step of treatment. Initial attention must be paid to cardiovascular support. If hypotension is present, fluid resuscitation and pressor support should be considered. Care should be taken to avoid fluid overload, however, especially in patients with impaired cardiac or renal function. Pulmonary artery catheterization is useful in selected patients to guide resuscitation.

Because intravascular hemolysis is an expected consequence of the infusion of ABO-incompatible marrow, some guidance can be obtained from published reports. Isoagglutinin titer clearly influences the clinical response to ABO-incompatible marrow infusion. In general, antibody titers below 64 are associated with mild or no reactions while high titers such as 1024 are associated with significant clinical reactions. The volume of incompatible red cells infused with the marrow also determines the magnitude of the response. One protocol reported to be successful in patients receiving major ABO-incompatible transplants involved preparatory hydration with 5% dextrose in one-half normal saline with 30 to 40 mEq sodium bicarbonate/L and 15 mEq potassium chloride/L at a rate of 3000 mL/m<sup>2</sup>/day, and 100 mL/m<sup>2</sup>. Mannitol 20% was given 1 hour before marrow infusion.<sup>76</sup> During marrow infusion, the infusion rate of fluids was increased to 4500 to 6000 mL/m<sup>2</sup>/day and additional mannitol was given at a rate of 30 mL/m<sup>2</sup>/hour for the next 12 hours. The rationale for this protocol was to maintain a high rate of urine output and prevent precipitation of hemoglobin in the renal tubules. All these patients had a preinfusion antibody titer no greater than 32. None experienced a clinical reaction to the infusion of approximately 120 to 160 mL of incompatible red cells.

The deliberate transfusion of ABO-incompatible red cells before transplantation has been employed to reduce

**Table 52-6.** Therapeutic Options in Hemolytic Transfusion Reactions

| Therapeutic Intervention       | Indication                                                                                                                  | Typical Dose                                                                                                                                                                   |
|--------------------------------|-----------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Hydration                      | <ul style="list-style-type: none"> <li>Prevent renal impairment</li> <li>Maintain urine output &gt;100 mL/hour</li> </ul>   | <ul style="list-style-type: none"> <li>Normal saline and/or 5% dextrose 200 mL/m<sup>2</sup>/hour</li> </ul>                                                                   |
| Alkalinization of urine        | <ul style="list-style-type: none"> <li>Prevent renal impairment</li> <li>Maintain urine pH &gt;7.5</li> </ul>               | <ul style="list-style-type: none"> <li>NaH<sub>2</sub>CO<sub>3</sub> 40 to 70 mEq in 1 liter 5% dextrose</li> </ul>                                                            |
| Diuresis                       | <ul style="list-style-type: none"> <li>Prevent renal impairment</li> </ul>                                                  | <ul style="list-style-type: none"> <li>Mannitol 20% 100 mL/m<sup>2</sup>*</li> <li>Furosemide 40 to 80 mg</li> </ul>                                                           |
| Vasodilation                   | <ul style="list-style-type: none"> <li>Increase renal blood flow</li> </ul>                                                 | <ul style="list-style-type: none"> <li>Dopamine 1 to 5 <math>\mu</math>g/kg/minute</li> </ul>                                                                                  |
| Anticoagulation                | <ul style="list-style-type: none"> <li>Treat intravascular coagulation</li> </ul>                                           | <ul style="list-style-type: none"> <li>Heparin 5 to 10 units/kg/hour, 0.15 to 0.25 unit per mL</li> </ul>                                                                      |
| Red cell exchange transfusion  | <ul style="list-style-type: none"> <li>Decrease load of incompatible red cells</li> </ul>                                   | <ul style="list-style-type: none"> <li>Exchange of one estimated red cell mass</li> </ul>                                                                                      |
| Plasma or platelet transfusion | <ul style="list-style-type: none"> <li>Treat hemorrhagic complications of disseminated intravascular coagulation</li> </ul> | <ul style="list-style-type: none"> <li>Platelets: 1 unit Platelets/10 kg (max. 6 units) or 1 unit Apheresis Platelets</li> <li>Plasma: 10 mL/kg Fresh Frozen Plasma</li> </ul> |
| Intravenous immunoglobulin     | <ul style="list-style-type: none"> <li>Prevent extravascular hemolysis<sup>†</sup></li> </ul>                               | <ul style="list-style-type: none"> <li>400 mg/kg</li> </ul>                                                                                                                    |

\*Ensure adequate renal function to prevent fluid overload from increased intravascular volume.

<sup>†</sup>Investigational. Not standard therapy.

isoagglutinin titers. In one such protocol, 1 incompatible RBC unit was given over 8 hours on each of 2 days immediately before transplant.<sup>39</sup> These patients were monitored in an intensive care unit and hydrated with normal saline and 5% dextrose (1:1 ratio) at a rate of 3000 mL/m<sup>2</sup>/day. Sodium bicarbonate was administered to maintain the urine pH above 7.0. Of the 12 patients reported in this series, isoagglutinin titers before transfusion ranged from 32 to 1024. One patient developed renal failure requiring hemodialysis for 17 days, but this resolved. In another series of deliberate administration of ABO-incompatible red cells, 35 patients received 50 to 150 mL of incompatible red cells before marrow transplantation.<sup>40</sup> All patients received prednisolone 250 mg, dimethindene 4 mg, and ranitidine 150 mg 30 minutes before the transfusion. No clinical reaction was observed in 23 patients (66%), while severe reactions (chest pain, headache or agues, and/or high changes of heart rate, blood pressure, and/or oxygen saturation) occurred in 4 patients (11%). The highest pretransfusion titer in this series was 64.

Because the severity and course of HTR is dictated by the load of incompatible red cells in circulation, exchange transfusion with antigen-negative blood may be considered. Although it is not appropriate to expose a patient to added risk of transfusion-related infectious disease if the hemolytic process is well tolerated, with a severe reaction to ABO incompatibility exchange transfusion might greatly reduce the chance of morbidity or death.

Early treatment of hypotension and DIC are the most important interventions to limit the extent of possible renal impairment. Maintenance of urine output with intravenous fluids and diuretics, such as mannitol or furosemide, early in the course of the reaction has been used successfully. However, if oliguria in the face of normovolemia is present, fluid loading is contraindicated. The use of vasopressor agents with direct vasodilatory effects on the renal vascular bed, such as low-dose dopamine (1–5 µg/kg/min) has been suggested, but is controversial.<sup>77,78</sup>

The prevention and treatment of DIC is also controversial. Heparin has been advocated by some authors as a treatment for DIC.<sup>79</sup> In addition, heparin may have a direct anticomplement effect, which limits intravascular hemolysis and the sequelae of complement activation.<sup>80</sup> An obvious drawback of heparin therapy, especially in the intraoperative or postoperative patient, is the potential for hemorrhage. Therefore, heparin should be reserved for patients with clear evidence of intravascular coagulation (thrombocytopenia, hypofibrinogenemia, presence of FDPs and D-dimers). The use of Fresh Frozen Plasma or platelet concentrates in DIC is also controversial, and transfusion of these components should be limited to those patients with active hemorrhage.

Most extravascular HTRs are not life-threatening and require no acute treatment. However, some patients with extravascular HTR may benefit from intravenous immunoglobulin (IVIG) infusion. A single dose of IVIG, 400 mg/kg infused within 24 hours of transfusion, has been used successfully to prevent transfusion reactions in alloimmunized patients for whom compatible

blood was not obtainable.<sup>41,81</sup> Five patients so treated did not experience transfusion reactions and had sustained increases in hematocrit. IVIG, 1 g/kg or 400 mg/kg, has also been used in the treatment of sickle cell HTR syndrome.<sup>82,83</sup>

The selection of blood components for a hemorrhaging patient undergoing HTR is a critical decision. The first consideration is that no patient should be allowed to suffer a fatal hemorrhage while a search for serologically compatible blood is undertaken. Second, red cells lacking known clinically significant antigens to which the patient currently has an antibody should be obtained, if at all possible. For instance, one should not reflexively issue group O-negative red cells to a patient known to have anti-e. When the specificity of the antibody causing the reaction is not known, the results of serologic tests performed up to that point in time must be considered, and clinical judgment exercised. Although the focus of attention in most HTRs is on red cells, care should be taken to avoid transfusion of type-incompatible plasma or platelets that may aggravate hemolysis, especially when ABO incompatibility is a possible cause. Undue haste in both serologic evaluation and decision making must be avoided, because human errors are often committed under pressure.

Future therapies that have not yet been subjected to clinical trials might be directed against inflammatory mediators produced during HTRs. HTRs have a similar clinical presentation to the systemic inflammatory response syndrome (SIRS) (eg, fever, tachycardia, tachypnea or hypoxemia, and leukocytosis).<sup>84</sup> Cytokine dysregulation is central to the pathophysiology of SIRS, in which elevated levels of TNF-α, IL-1, and IL-6 are associated with mortality.<sup>85,86</sup> These considerations suggest that TNF-α or IL-1 blockade may be beneficial in the treatment of HTR. Presently, there are three TNF-α blocking agents marketed in the United States; etanercept (Enbrel, Amgen, Thousand Oaks, CA), a soluble p75 TNF-α receptor fusion protein to the Fc portion of IgG; infliximab (Remicade, Centocor, Malvern, PA), a chimeric (mouse/human) TNF-α antibody; and adalimumab (Humira, Abbott Laboratories, Abbott Park, IL), a fully human monoclonal antibody. Anakinra (Kineret, Amgen) is recombinant human IL-1ra, which is also approved by the FDA. Unfortunately, clinical trials of these agents in SIRS have been rather disappointing, with most trials not showing improved outcome. Inhibitors of complement show promise in the treatment of HTR. Human recombinant soluble form of complement receptor 1 (sCR1) has been shown to inhibit complement-mediated red cell destruction in vitro and in a mouse model of hemolysis.<sup>87</sup> It should be noted that there are no data at present regarding the safety or effectiveness of drugs that inhibit complement or cytokines in HTR.

## Prevention

Much of the activity in blood banks is directed toward the prevention of HTRs. Proper performance of donor unit typing, pretransfusion testing, antibody identification, and crossmatching are critical and are covered in Chapter 5.



Because human errors are the most common cause of severe HTR, administrative systems designed to analyze errors and prevent future recurrence are the most important protective measure. An event reporting system specifically designed for transfusion services, MERS-TM, has been developed.<sup>88</sup> It allows for the recognition and analysis of errors, determination of patterns of errors, and monitoring for changes in frequency after corrective action is implemented. In one application of MERS-TM, high severity events with the potential for patient harm were discovered to account for 241 (5%) of the 4670 events over a 47-month period.<sup>89</sup>

Proper identification of the transfusion recipient and pretransfusion blood specimen is the single most important aspect of the prevention of HTR. Every transfusion service must establish and enforce the procedures to be followed in its institution. At a minimum, these procedures should include permanent and unique identification of each patient using a permanent identification method such as a wristband, confirmation of the proper labeling of blood specimens by comparison to the wristband, and confirmation of the patient identification before starting the transfusion. Deviation from institutional policies on patient and specimen identification should be taken very seriously.

Because proper identification of the transfusion recipient is crucial to preventing HTRs, barrier systems that are intended to physically prevent the transfusion of blood without correct identification of the patient have been devised. One such system uses a plastic lock that is preset to a three-letter code at the time of blood component issuance by the blood bank. Before the unit can be administered, the identical code must be entered to unlock the system. Use of this system in one hospital over the course of 1 year detected two misidentified pretransfusion blood samples, and prevented one attempt to transfuse blood to the wrong patient.<sup>90</sup> Use of a special wristband for identification of transfusion recipients may prevent some errors. Such systems generally have a unique identifier on the wristband that is only used by the blood bank. A report of the use of one system over a period of 17 years found that potentially ABO-incompatible transfusions were avoided in five of 411,705 samples typed.<sup>91</sup> A report of the use of another identification system found that of 2198 cases, two potentially ABO-incompatible transfusions were avoided.<sup>92</sup> An alternative system uses bar codes on patient wristbands, blood sample tubes, blood component bags, and nurses' identification badges; and point-of-care reading devices to verify identity.<sup>93</sup> Radio frequency identification devices also have great promise for reduction error. These tools require investment in information technology, but can be integrated with other systems, such as medication administration, for overall enhancement of patient safety. However, sole reliance on such a device to prevent incorrect administration of blood may undermine other more important steps in proper patient identification.

Confirmation of the recipient's ABO type by point-of-care testing before transfusion is a possible strategy for avoiding AHTR. In principle, seven out of eight ABO-incompatible transfusions could be thus avoided. However, bedside testing is subject

to analytic and interpretive errors. At one institution performing bedside ABO confirmation, 13 ABO-incompatible RBC transfusions occurred in 8 years.<sup>94</sup> Of these, an error in bedside ABO testing occurred in seven cases. User inexperience is the most important factor in bedside testing errors, although there are device factors as well.<sup>95</sup>

In situations where patient identification error has caused HTR, immediate consideration should be given to the possibility that another patient has been involved in the misidentification, and may, too, be at risk of receiving incompatible blood. This is especially likely if there are two patients with similar names or if two samples are received simultaneously from the same patient care location. Identification of such errors can prevent a second hemolytic reaction.

The selection of donor units lacking antigens corresponding to alloantibodies is essential for the prevention of HTR, but whether phenotype matching of donors and recipients is an appropriate strategy for the prevention of alloimmunization and HTR in the nonimmunized patient is controversial. Arguments in favor of this practice have been put forth in the setting of sickle cell disease where, because of ethnic gene pool diversity, there is often a mismatch between the donor and recipient phenotypes. Examination of alloimmunization rates among children in one urban area indicated that children with non-European ethnic origins had a 42.9% incidence of alloimmunization compared to 17.6% in patients of European ancestry.<sup>96</sup> This was not simply caused by variation in transfusion rates, because the patients of European ethnicity received more transfusions than the others. In another study that compared sickle cell patients to those with other forms of chronic anemia, 30% of the patients with sickle cell anemia were alloimmunized, in contrast to 5% in the group of patients with other forms of anemia.<sup>97</sup> Of the 32 alloimmunized patients with sickle cell anemia, 17 had multiple antibodies and 14 had DHTRs. Although many institutions have not performed phenotype matching for sickle cell patients in the past, it is becoming commonly accepted practice.<sup>98,99</sup>

Phenotype matching may possibly prevent some HTRs in other multitransfused patient populations. In a retrospective study of patients with at least one red cell antibody who received subsequent transfusions, 21% of 653 patients produced additional antibodies.<sup>100</sup> Patients with hematologic disease or malignancy, warm-reacting autoantibodies, antibodies to high- or low-frequency antigens, and those receiving prophylactic antigen matching were excluded. Of those who formed additional antibodies, 57% did so after one transfusion episode of a median of 2 RBC units. The authors predicted that matching for Rh, Jk<sup>a</sup>, and Fy<sup>a</sup> could have prevented the formation of 83% of new antibodies. However, whether prevention of serologic reactions will prevent hemolytic reactions is not clear. In this study, 1% to 10% of potential donors would have been available for 3% of patients and more than 10% of potential donors would have been available for 61% of patients, had phenotype matching been performed. However, the frequency of suitable donors may be different in other locations.

Hemolytic transfusion reactions could be avoided entirely if the responsible antigens on the red cell surface could be removed or camouflaged. Experimental work has suggested that treating red cells with polyethylene glycol (PEG) might be an effective means of camouflaging antigens.<sup>101</sup> PEG modification appears to work by creating a sphere of hydration around the red cell that effectively excludes IgG or IgM from coming into contact with antigenic structures on the membrane surface. PEG-treated red cells have properties of size, shape, intracellular ion content, and oxygen binding that are identical to untreated red cells. However, PEG-treated red cells have a low shear viscosity compared to normal red cells. This also may be advantageous in sickle cell disease in which increased blood viscosity within capillaries can result in occlusive crises.

The effectiveness of PEG modification is dependent on the molecular weight and branching characteristics of PEG molecules and the chemistry of covalent attachment.<sup>102</sup> Use of a dichlorotriazine derivative of 5-kD PEG results in complete inhibition of direct agglutination by anti-D. However, such cells are still agglutinated by anti-D in the indirect antiglobulin test. A and B epitopes are partially, but not completely, masked. In contrast, red cells coated with branched chain 10-kD PEG after treatment with succinimidyl-propionate-modified 20-kD PEG are not agglutinated by anti-A, anti-B, and anti-D. Treatment of red cells with maleimidophenyl-PEG 5kD and 20kD can inhibit agglutination by Rh and ABO antibodies.<sup>103</sup> However, preliminary data from human trials with PEG-treated red cells have shown that antibodies to the modified red cells resulting in accelerated clearance occur in some recipients, which may significantly limit the usefulness of this technique (G. Garratty, personal communication).

Group A and group B red cells can be converted to group O cells by enzymatic cleavage of terminal determinant saccharides with  $\alpha$ -N-acetylgalactosaminidase or  $\alpha$ -D-galactosidase.<sup>104-106</sup> The use of such technology for large-scale conversion of red cells raises the possibility that acute HTRs from ABO incompatibility may be completely avoidable in the future. However, there are issues with regard to completeness of antigen removal and the possibility of exposure of neoantigens by enzymatic treatment. Treatment of red cells with  $\alpha$ -N-acetylgalactosaminidase results in rapid loss of A epitopes binding *Dolichos biflorus* lectin.<sup>104</sup> Inhibition of complement-mediated hemolysis is somewhat slower. However, the epitopes of A antigen that react with human source anti-A are relatively resistant to enzymatic degradation. Additionally, there are differences in the enzymatic sensitivity of A epitopes on the red cell membrane. Glycosphingolipids with short oligosaccharide chains display the greatest resistance to enzymatic treatment.

Work on enzyme-converted group O (ECO) red cells from group B red cells has advanced to the stage of the clinical trials.<sup>107,108</sup> An initial trial of a 2-unit transfusion of ECO RBCs to group O subjects demonstrated good 24-hour posttransfusion survival (95%), with a half-life of 29.5 days.<sup>107</sup> There was no clinical or laboratory evidence of hemolysis. A subsequent study

with larger volume transfusions had similar results. Subjects who received a second transfusion did not show evidence of alloimmunization or increase in anti-B titer.<sup>108</sup> In another study, occasional serologic incompatibility with enzyme-converted red cells was seen, as well as transient positive DAT and increases in anti-B titer after transfusion. However, no adverse effects of whole unit transfusion were seen, and normal posttransfusion red cell survival was demonstrated in the face of incompatibility.<sup>109</sup> A screening technique has recently been used to discover several novel exoglycosidases with high specific activity for the removal of A and B determinants at neutral pH.<sup>110</sup> Complete conversion of whole RBC units from group A and group B to group O, as indicated by no reactivity with licensed typing reagents, was achieved with low enzyme concentrations. Although this technique has not yet been subjected to clinical trials, it is promising as a feasible method of ECO production on a clinical scale.

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## Summary

Although the prevention of HTRs continues to be a major focus in transfusion medicine, advances in serology and transfusion service practices have significantly reduced their incidence. Simultaneously, advances in the understanding of the pathophysiology of HTRs have given us insights to help guide the management of patients undergoing reactions. It is conceivable that technological advances in red cell modification and oxygen-carrying solutions will significantly change red cell transfusion practice in the future and virtually eliminate the occurrence of HTR. Until such time, however, HTRs will remain a major adverse consequence of blood transfusion.

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## Disclaimer

The author has disclosed no conflicts of interest.

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# Febrile, Allergic, and Nonimmune Transfusion Reactions

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This chapter reviews a variety of acute, nonhemolytic, and non-infectious transfusion reactions, the most common of which are febrile, nonhemolytic transfusion reactions (FNHTRs) and allergic reactions. Other acute, nonhemolytic reactions are reported less frequently and include transfusion-related acute lung injury (TRALI) and anaphylactic or anaphylactoid reactions. Additional acute adverse effects can occur in massive transfusion because of the large volume of blood components transfused over a short period. The complications of massive transfusion include dilutional coagulopathy, hypothermia, citrate toxicity, and electrolyte disturbances among others. Some patients cannot tolerate the acute increase in intravascular blood volume caused by transfusion and experience the complications of transfusion-associated circulatory overload (TACO). Acute reactions can be caused by the toxicity of chemicals that leach into blood components from blood storage containers or filters or by chemicals added to improve storage conditions, such as dimethyl sulfoxide (DMSO). Other reactions are caused by endogenous mediators generated in the blood during filtration, processing, or storage, such as bradykinin-mediated hypotensive reactions. It is important that these complications of transfusion be recognized by patient care teams and blood bank personnel, and that appropriate treatments and preventive measures be instituted for patient safety and well-being.

## Febrile Nonhemolytic Transfusion Reactions

### Description

An FNHTR is commonly defined as an increase in body temperature of 1°C or more that occurs during or within several hours of transfusion and is unrelated to hemolysis, sepsis, or other known causes of fever. The use of a 1°C increase in body

temperature as a threshold for defining an FNHTR avoids undue concern over small fluctuations in body temperature unrelated to transfusion that do not justify discontinuation of transfusion and follow-up investigation. Many FNHTRs begin with the patient feeling uneasy and experiencing chills. In mild reactions, the signs and symptoms do not progress. Chills with or without an increase in body temperature can be classified as an FNHTR if other possible causes of chills are unlikely and the time course of the reaction correlates with the transfusion. In the most severe reactions, patients may experience rigors (severe shaking chills) or a fever elevation of 2°C or more over baseline. Although signs and symptoms usually are limited to chills and fever, some patients may also rarely experience more severe symptoms such as headache, nausea, and/or vomiting.

The fever of FNHTR usually persists no more than 8 to 12 hours after the start of transfusion. If fever persists 18 to 24 hours or longer, it is unlikely to be transfusion related. Generally, FNHTRs are self-limited and have no sequelae. However, elderly patients, patients with compromised cardiovascular status, or critically ill patients are at risk of cardiorespiratory complications associated with FNHTR. Because fever increases oxygen demand and consumption an estimated 13% for every 1°C over 37°C and shivering increases oxygen demand approximately 300%, FNHTRs can aggravate preexisting cardiac, pulmonary, and cerebrovascular insufficiency. Therefore, prompt recognition and antipyretic management of FNHTRs can be very beneficial.

An FNHTR almost always is associated with transfusion of cellular blood components, such as red cells, platelets, and granulocyte preparations and less commonly with noncellular components, such as plasma and cryoprecipitate. The incidence of FNHTR varies widely and median rates have been reported as higher for platelets (4.6%) than for red cells (0.33%).<sup>1</sup> The reaction risk of blood components, however, varies according to numerous factors, such as method of preparation of the blood component (eg, leukocyte reduction, storage time, medications, patient and donor characteristics, monitoring practices, and many others). These factors vary among different geographic regions and medical centers. In addition, rates based on reactions

*Rossi's Principles of Transfusion Medicine, 4th edn.* Edited by T. L. Simon, E. L. Snyder, B. G. Solheim, C. P. Stowell, R. G. Strauss and M. Petrides. © 2009 AABB published by Blackwell Publishing, ISBN: 978-1-4051-7588-3.

reported to blood banks are lower than those based on systematic surveillance of responses to all transfusions.

The cause of higher FNHTR reaction rates with platelets is not entirely known. Pools of whole-blood-derived concentrates have been reported to cause a higher rate of FNHTR than apheresis platelets.<sup>2,3</sup> Platelet concentrates prepared from platelet-rich plasma are commonly used in the United States. These platelet-rich plasma concentrates are reported to cause a higher rate of FNHTR than are platelet concentrates prepared from the buffy-coat technique. Longer platelet storage times are also associated with higher rates of FNHTR.<sup>4-6</sup> Reactions also are more frequent among certain recipients, such as multitransfused patients or multiparous women who have developed leukocyte or platelet alloantibodies.

### Etiology

An FNHTR appears to be part of the systemic inflammatory response syndrome (SIRS) provoked in transfusion recipients by the immune challenge of transfusing foreign cells or infusing soluble inflammatory mediators present in stored blood components. The term *systemic inflammatory response syndrome* was coined to describe the constellation of observed body responses to various insults, such as infection, trauma, burns, and ischemia. It is defined as the presence of two or more of the following: body temperature more than 38°C or less than 36°C; heart rate more than 90 beats/minute; tachypnea (respiratory rate >20 breaths/minute or PaCO<sub>2</sub> less than 32 mm Hg); white cell count more than 12,000/μL or less than  $4 \times 10^9/L$ , or more than 10% immature neutrophils (band forms). Although a mild FNHTR may not completely fulfill these criteria, FNHTR is nevertheless an inflammatory response.

Exogenous pyrogens such as lipopolysaccharide (LPS) and pyrogenic cytokines initiate a series of responses leading to hyperthermia. These responses include rapid muscle contractions that cause shivering, rigors, and an increase in heat generation. Heat conservation is achieved through cutaneous vasoconstriction, which also contributes to the sensation of a chill. Perceived chills lead to behavioral changes that can further increase body temperature. For example, the patient may cover up, and the result is inhibition of heat dissipation.

An FNHTR appears to have two possible underlying causes: 1) the more “classical” pathway of infused antigens, such as leukocytes, that stimulate the in-vivo generation of cytokines in the recipient and 2) the infusion of pyrogenic cytokines or other inflammatory response mediators (eg, activated complement proteins, LPS, or neutrophil-priming lipids) that accumulate in the plasma portion of cellular blood components during storage.<sup>7,8</sup> A third cause of fever, infusion of blood components contaminated with bacteria or bacterial products, will produce a febrile response but is not usually categorized as a FNHTR but rather as a bacterial septic reaction, if recognized. The common pathway by which these different stimuli induce posttransfusion fever has been attributed to an increase in circulating pyrogenic cytokines in the recipient, such as interleukin-1β (IL-1β),

IL-6, and tumor necrosis factor α (TNF-α). Pyrogenic cytokines induce fever by mediating upregulation of the thermostatic set point for body temperature in the thermoregulatory center of the hypothalamus. This mechanism is supported by the association of febrile reactions with a specific cytokine polymorphism *IL1RN\*2.2* genotype.<sup>9</sup>

Recently, research has lead investigators to a more complex model of fever generation, building on a model where cytokines principally induce fever to one where central nervous system stimulation by prostaglandin E2 (PGE2) is pivotal. Alternative hypotheses have resulted from the finding that a febrile response to LPS occurs even with blockade of either IL-1 or TNF-α and that the presence of circulating cytokines lags behind the development of fever.<sup>10</sup> Research has found that LPS-induced C5a production via complement activation results in rapid peripheral PGE2 production.<sup>11</sup> In addition, LPS binds to toll-like receptor 4 and induces cytokine production, leading to a two-phase rapid and delayed febrile response. Hyperthermic stimuli compete with hypothermic stimuli to achieve a central thermal balance point that may elevate or decrease based upon physiologic stimuli.<sup>12</sup> Central to the febrile response is the presence of EP3 prostaglandin receptors that bind PGE2 in the hypothalamus (Fig 53-1).<sup>13</sup> These newer models of the fever response provide possible explanations for why FNHTRs continue to occur despite prestorage leukocyte reduction that minimizes cytokine accumulation in storage. Clinical evidence supports the hypothesis that febrile reactions can be caused by noncytokine hyperthermic stimuli present in cellular transfusion products.<sup>14</sup> The active field of febrile response research shows promise for the development of more targeted antipyretic medications that may eventually lead to the extinction of FNHTRs.

### Alloimmunization to Leukocytes or Platelets

Transfusion recipients at greatest risk of an FNHTR are those with leukocyte or platelet antibodies who receive transfusions with blood components containing large numbers of passenger leukocytes or platelets.<sup>15,16</sup> Less frequently, donor antibodies to leukocytes, present in the plasma portion of blood components, are associated with FNHTRs. The implicated antibodies most often have HLA specificity, although they also may be platelet- or granulocyte-specific. A minimum of approximately  $1 \times 10^7$  leukocytes per unit of Red Blood Cells (RBCs) appears necessary to cause an FNHTR, although this number varies among individuals.<sup>17,18</sup> The role of donor leukocytes in FNHTR is supported by the finding that decreasing the leukocyte content of blood components below this threshold reduces the incidence of FNHTRs.

A variety of mechanisms are possible by which antibody-leukocyte or antibody-platelet interactions cause fever. For example, donor monocytes may be activated and secrete pyrogenic cytokines when recipient antibodies bind to them. An alternative explanation is that immune complex formation between recipient antibodies and donor leukocytes or platelets leads to generation of activated complement components such as C5a, which stimulate the production of PGE2.

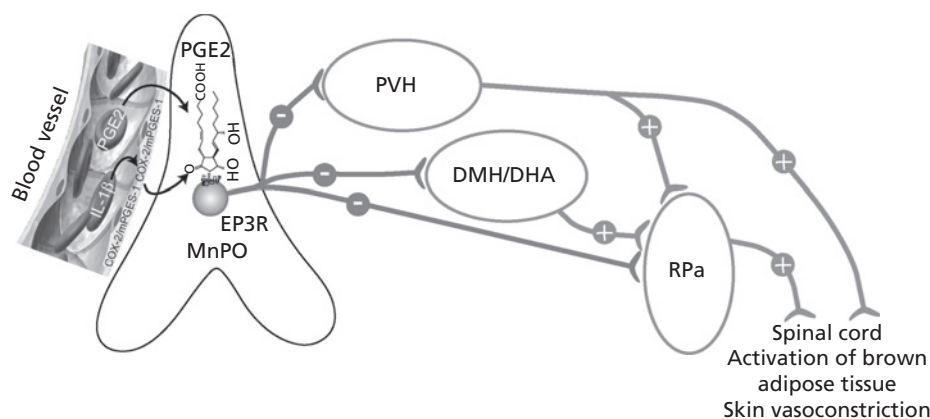


**Figure 53-1.** A model for neuronal action of the EP3 receptors (EP3Rs) in the fever response after systemic immune challenge. In response to lipopolysaccharides or cytokines, prostaglandin E2 (PGE2) is generated by macrophages of the liver and lung and endothelial cells lining venules in the brain. PGE2 is transported or diffuses across the blood-brain barrier and acts on EP3Rs in the median preoptic area (MnPO). These are hypothesized to be GABAergic

neurons that inhibit thermogenic systems in the hypothalamic paraventricular nucleus (PVH), the dorsomedial nucleus/dorsal area (DMH/DHA), and the raphe pallidus nucleus in the medulla (RPa). These thermogenic nuclei activate sympathetic preganglionic neurons in the spinal cord, resulting in cutaneous vasoconstriction and activation of brown adipose tissue that ultimately raise body temperature. Used with permission from Lazarus et al.<sup>13</sup>

### Storage-Generated Cytokines

Antibodies to leukocytes or platelets do not appear to account for all FNHTRs, particularly those caused by platelet transfusions. For example, some patients with no history of transfusion or pregnancy experience an FNHTR to their first transfusion of platelets.<sup>2</sup> It is unlikely that these reactions are mediated by recipient leukocyte or platelet antibodies because these recipients have no previous exposure to foreign cells. In addition, the rate of FNHTRs to platelet transfusion increases with increasing blood bank storage time of the transfused platelet concentrate.<sup>4-6</sup> This indicates that time-dependent change occurs in the platelet concentrate during storage that has a role in stimulating an FNHTR in some patients. Furthermore, febrile reactions still occur with the use of prestorage leukocyte reduction.<sup>19,20</sup> In some cases, this is the result of inappropriate filter use or filter failure. However, this observation also supports the possibility that a substance or substances in the plasma portion of blood components not removed by filtration may be responsible for mediating at least some FNHTRs. The discovery that proinflammatory cytokines accumulate in the plasma portion of platelet concentrates may account for many of these findings.

A variety of leukocyte-derived, proinflammatory cytokines, including IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ , macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ), and growth-related oncogene  $\alpha$  (GRO- $\alpha$ ), are generated and accumulate in the plasma portion of platelet concentrates during storage.<sup>8,21-23</sup> Extracellular levels of these cytokines generally increase with increasing component storage time and are roughly proportional to the passenger leukocyte content of the blood component bag. Prestorage or early-storage leukocyte reduction (within 1 to 2 days of collection) prevents or greatly reduces generation of these cytokines. Because they have pyrogenic activity, many of these cytokines (if present in high enough concentration) can induce febrile responses in

transfusion recipients. Elevated levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in the plasma portion of platelet concentrates correlate positively with the occurrence of an FNHTR. Some studies have shown that IL-6 levels in the plasma portion of platelet concentrates correlate best with the occurrence of FNHTR. In one study, chills, fever, or both occurred more frequently after infusion of the plasma portion of the platelet concentrates than after infusion of the cellular portion containing platelets and leukocytes.<sup>21</sup> The plasma portions that caused an FNHTR contained higher levels of IL-1 $\beta$  and IL-6 than did those that did not cause chills or fever. These data support the role of the plasma portion of platelet concentrates as a source of inflammatory mediators and as a possible stimulus of FNHTR in some transfusion recipients. Although levels of a variety of proinflammatory cytokines in the plasma portion of platelet concentrates correlate with the occurrence of FNHTRs, it is unknown which, if any, of these actually mediate FNHTRs. Other inflammatory mediators, such as C5a and biologically active lipids, accumulate in platelet concentrates during storage and also may be implicated.<sup>24,25</sup>

Platelet-derived cytokines, such as CD40L (CD154), CCL5 (RANTES), transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1), CXCL4 (platelet factor 4; PF4), CXCL8 (IL-8), and MIP-1 $\alpha$  are present in the plasma portion of platelet concentrates and apheresis platelets. All platelet-derived cytokines accumulate during platelet storage and CD40L has been associated with clinical FNHTRs.<sup>26</sup> These cytokines are not known to be directly pyrogenic, but they stimulate the synthesis of proinflammatory mediator, IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$ . Because RANTES can activate basophils and mast cells and stimulate histamine release, it may play a role in mediating some allergic reactions (see later, Allergic Reactions).<sup>27</sup> RANTES data are conflicting as to the extent of accumulation during storage; however, it seems that some increase in levels does occur.<sup>28</sup>



Some proinflammatory cytokines, such as IL-1 $\beta$  and IL-8, have been detected in the supernatant portion of stored RBCs, although at much lower levels than in platelet concentrates.<sup>29</sup> Because the cold storage temperature of RBC units, 1-6°C, has an inhibitory effect on cellular metabolism, the capacity of passenger leukocytes in RBC units to synthesize and secrete cytokines is less than those in platelet concentrates. As a result, the levels of cytokines in RBC units appear to be too low to mediate significant physiologic reactions.

The stimulus for cytokine generation during storage of blood components remains unknown. Measurements of cytokine messenger RNA levels and total cytokine levels (intracellular plus extracellular) in platelet concentrates indicate that accumulation of leukocyte-derived cytokines is caused in part by new synthesis and secretion. The stimulus for synthesis and secretion of leukocyte-derived cytokines may be, for example, contact activation of monocytes after these cells interact with the plastic of the storage containers or tubing.<sup>30</sup> Other possibilities include the direct stimulatory effects of C5a on PGE2 or the stimulatory effects of activated complement components on monocytes or other leukocytes in the blood component bag. The presence of platelet-derived cytokines (such as CD40L, RANTES, and TGF- $\beta$ 1) in the plasma portion of platelet concentrates likely results from their release from preexisting stores, because the biosynthetic activity of platelets is limited.

### Bacterial Contamination of Blood Components

An FNHTR may result from infusion of a blood component contaminated with bacteria or bacterial products such as LPS. Unless a Gram's stain and bacterial cultures are performed, mild septic transfusion reactions characterized by only fever and chills are likely to be classified clinically as FNHTR (see Chapter 49).<sup>31,32</sup>

Transfusion reactions caused by contaminating bacteria, whether mild or severe, are manifestations of the SIRS, described earlier. Proinflammatory cytokines (such as IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$ ) are implicated in the pathogenesis of SIRS associated with sepsis.<sup>33</sup> If bacterial contamination of blood components has occurred, the greatest source of cytokines is likely the transfusion recipient's cells stimulated by the infused bacteria or bacterial products. However, cytokine production by leukocytes in the component bag during storage stimulated by bacteria or bacterial products also may contribute to the reaction.

### Diagnosis

As a routine part of the transfusion procedure, the vital signs of transfusion recipients (pulse, temperature, and respiratory rate) should be measured immediately before transfusion and at intervals during and soon after transfusion. The patient should be watched closely, particularly in the first 30 to 60 minutes of transfusion, for the onset of chills, shivering, or rigors, which often precede a fever.

A transfusion reaction is a possibility if chills, fever (1°C or more over pretransfusion temperature), or both develop any time during transfusion or up to several hours after the transfusion

has ended. A febrile response to transfusion, however, is not specific for an FNHTR. For example, a fever may be the early manifestation of a more serious acute hemolytic or septic transfusion reaction. When a patient has a febrile reaction to transfusion, an evaluation to rule out hemolysis and possibly bacterial contamination should be undertaken promptly. Nursing staff should stop the transfusion immediately and notify the physician caring for the patient. They should verify that the identity of the transfusion recipient, based on the patient's identification bracelet and verbal confirmation with the recipient, if possible, matches that of the intended recipient, as indicated on the blood component tag. All containers and transfusion sets should be sent to the laboratory along with a posttransfusion blood specimen and a report that summarizes the clinical reaction. The clinical team should also have verbal communication with the blood bank staff to ensure that the postreaction blood specimen, component bag, and infusion set are received by the blood bank as soon as possible.

Investigation of a febrile reaction in the blood bank generally begins with a recheck of the records for clerical errors. The posttransfusion serum must be visually evaluated for hemolysis and should be compared with the pretransfusion serum. A direct antiglobulin test must be performed on the posttransfusion blood specimen and ideally on a pretransfusion specimen for comparison. The ABO grouping of the patient sample and the donor unit should be repeated. When suggested by the preliminary serologic results, the crossmatch may be repeated for RBC transfusions to confirm patient-donor compatibility. The results of these tests confirm or exclude a hemolytic transfusion reaction as the basis for the fever. When a septic reaction is highly suspected, for example if the patient arrives with a high fever (2°C or more) or accompanying hypotension, the bag contents should be examined by means of a Gram's stain and culture for bacterial contamination. Blood cultures also should be obtained from the transfusion recipient's posttransfusion blood specimen to correlate bacteremia with the same organism that may be detected in the blood component bag. Most blood banks do not test for HLA-specific, platelet-specific, or granulocyte-specific antibodies in the recipient's serum as possible causes of an FNHTR. Identification of these antibodies and pyrogenic cytokines is reserved for specialized laboratories and does not play a role in the immediate evaluation of most reactions.

The patient is examined by the primary team and the blood bank physician to determine whether associated symptoms or circumstances can explain the fever, such as drug reactions, sepsis, or other inflammatory conditions unrelated to transfusion. The time course of the development and resolution of fever should be examined in relation to the transfusion. In cases in which the transfusion recipient has a fever at the start of transfusion or is experiencing intermittent spiking fevers, a posttransfusion increase in body temperature can be difficult to interpret. In such cases *possible FNHTR* may be the most definitive diagnosis that can be made. An FNHTR is a diagnosis of exclusion, arrived at by means of eliminating the possibility of immune hemolysis, bacterial contamination of the blood component, TRALI, or other causes of fever unrelated to transfusion.

## Treatment

The transfusion should be stopped immediately. The intravenous line should be kept open with normal saline solution to provide ready access for the possible infusion of crystalloid and intravenous medication in case the fever is a sign of a more serious hemolytic or septic reaction. Most patients, however, should be reassured that febrile transfusion reactions usually are harmless and that the fever typically responds to antipyretic therapy. The antipyretic agent of choice is acetaminophen (adults, 325 to 650 mg orally; children, 10 to 15 mg/kg orally or rectally). Aspirin and nonsteroidal antiinflammatory drugs are contraindicated in the treatment of many transfusion recipients, such as those receiving platelets. Unless the patient has signs of an allergic reaction, such as urticaria, erythema, or pruritus, antihistamines are not indicated in the management of FNHTR. However, it is not unusual for physicians to prescribe both an antipyretic and an antihistamine in combination for mild reactions.

Patients occasionally develop rigors (severe shaking chills) after a transfusion and meperidine had been a mainstay therapy for many years. Because shivering can increase oxygen demand significantly, it is important to control the shaking chills, particularly for patients with cardiac or respiratory insufficiency. When administered to adults at doses of 25 to 50 mg intravenously, meperidine remains a very effective treatment for rigors. Meperidine is effective in rapidly arresting rigors through mechanisms not clearly understood. Unfortunately, meperidine has fallen out of favor with some hospitals because of unacceptable central nervous system toxicities and other downsides. Use of meperidine is generally contraindicated in the care of patients with renal failure because of accumulation of the proconvulsant metabolite normeperidine. Use of meperidine also is contraindicated in the care of patients who have taken monoamine oxidase inhibitors within the previous 14 days because of the risk of serotonin syndrome (excess serotonin activity). Toxicities secondary to metabolite accumulation, short half-life, and higher equianalgesic dose compared to morphine has decreased interest and formulary availability for this opioid.<sup>34</sup> On the basis of anecdotal evidence, morphine may be slightly less efficacious for the treatment of rigors, but its safety profile is more acceptable when given as a one-time dose of 2 to 4 mg intravenously.

After symptoms of an acute febrile reaction have been treated and the patient has been stabilized, any unused portion of the blood component should be returned to the blood bank and not transfused, even if blood bank testing rapidly rules out hemolysis. This is because no quick test is currently available that is sensitive enough to also rule out with confidence bacterial contamination of the blood component. The use of Gram's stain helps detect heavily contaminated units (with detection limits not less than  $10^6$  organisms per mL), and lower levels of contamination may be missed. If the febrile reaction is caused by bacterial contamination of the component bag, restarting transfusion of the same component can cause a severe and even fatal septic transfusion reaction as more bacteria or bacterial products are infused. For this reason, a new blood component unit should

be used if transfusion is still needed after the patient's condition has been stabilized.

Restarting transfusion with the same platelet preparation that caused an FNHTR may be considered as a last resort in the treatment of patients who repeatedly have mild and uncomplicated febrile responses to platelet transfusions despite prophylactic measures.<sup>35</sup> In this situation, it may be necessary to cautiously complete the transfusion with the same component, because the patient is likely to react to other platelet preparations. The patient should have no other symptoms and the fever should be less than 1.5°C over the pretransfusion baseline. A septic reaction caused by bacterial contamination of a unit is generally the major concern in this setting. Hemolysis of either donor or recipient red cells usually is not significant because of the small amount of red cells and plasma in platelet preparations. The transfusion should generally not be restarted for at least 30 minutes as a precaution to allow other possible signs or symptoms of a serious reaction to develop. High transfusion-related fevers, such as a 2°C increment or more, are more likely to be associated with septic reactions and should preclude restarting the transfusion. However, lesser fevers do not rule out bacterial contamination of the blood component. If the transfusion is restarted, the patient should be made as comfortable as possible with appropriate antipyretic therapy, as described earlier. The transfusion should proceed slowly and the patient observed closely for further signs of a reaction or further temperature elevation throughout the transfusion, which should be stopped if symptoms recur. Restarting transfusion of a blood component that has caused an FNHTR should not be routine.

## Prevention

### Premedication

Premedication with acetaminophen but not diphenhydramine should be considered for patients with a history of FNHTR. Patients who have no history of FNHTR do not need premedication. Despite a number of studies showing no benefit to premedication in preventing transfusion reactions, the practice remains commonplace in many institutions.<sup>36</sup> Premedication with the glucocorticoid hydrocortisone sodium succinate (adults, 100 mg intravenously) may be useful in the care of reaction-prone patients when an antipyretic agent alone is ineffective. Glucocorticoids have antiinflammatory effects that may help prevent or reduce the severity of FNHTRs. For example, they inhibit the enzyme phospholipase A<sub>2</sub>, thereby blocking production of arachidonic acid and its metabolites such as PGE<sub>2</sub>, a key mediator of fever. Glucocorticoids also inhibit synthesis of pyrogenic cytokines, such as IL-1 and IL-6. A variety of glucocorticoids other than hydrocortisone are available. However, hydrocortisone has the advantage of being a short-acting glucocorticoid (biologic half-life, 8 to 12 hours), and it induces a shorter period of immunosuppression than do many other glucocorticoid preparations. Because glucocorticoids generally act through changes in gene expression, hydrocortisone should be administered at least 4 to 6 hours before transfusion so that its antiinflammatory action has time to take effect.

### Rate of Infusion

Slowing the speed of infusion of a blood component can possibly prevent or decrease the severity of FNHTR. The rate of increase in body temperature in FNHTR caused by leukocyte alloimmunization appears to be directly related to the rate of infusion of leukocytes in the blood components.<sup>18</sup> A slower rate of infusion is of theoretic advantage in decreasing the severity of reactions caused by bacterial contamination or storage-generated cytokines. Slower infusion avoids a sudden bolus of bacterial toxins or cytokines that may provoke an immediate and possibly massive inflammatory response.

### Leukocyte Reduction

The prophylactic transfusion of leukocyte-reduced components in the treatment of patients receiving repeated transfusions is effective in avoiding alloimmunization to leukocytes, which is one of the major causes of FNHTR. Leukocyte-reduced blood components ideally should be transfused to such patients beginning with the first transfusion. Leukocyte reduction is effective in the care of patients already alloimmunized to leukocytes, because FNHTRs in these patients are directly related to the number and rate of infusion of passenger leukocytes. The threshold number of white cells associated with the development of an FNHTR generally ranges from  $0.25 \times 10^9$  to  $2.5 \times 10^9$ .<sup>18</sup> The removal of approximately 90% of leukocytes ( $10^{10}$ ), which usually leaves less than  $5 \times 10^8$  white cells per RBC unit, is sufficient to prevent most FNHTRs.<sup>17,37</sup> For that reason, leukocyte reduction for the purpose of preventing FNHTRs often is defined as decreasing the passenger leukocytes to less than  $5 \times 10^8$  per transfusion. Leukocyte reduction of blood components can be performed either at the time of component preparation (prestorage leukocyte reduction) or immediately before transfusion (poststorage leukocyte reduction). Poststorage leukocyte reduction by means of filtration can be performed in the blood bank before distribution of the component for transfusion or during administration of blood components. The latter often is called *bedside leukocyte reduction*.

Leukocyte-reduced RBC units have in the past been prepared by various poststorage techniques, including simple centrifugation with buffy-coat removal, saline washing, and deglycerolization of frozen RBCs.<sup>38,39</sup> Saline-washed and frozen deglycerolized RBCs are rendered leukocyte reduced because approximately 1 to 2  $\log_{10}$  leukocytes are removed by repeated centrifugation and washing steps on automated cell washers. Filtration of RBCs units through microaggregate filters designed to remove microaggregate debris more than 40 microns in diameter after an extra centrifugation step or after centrifugation and cooling (spin, cool, filter) also has been shown to reduce leukocytes in RBC units sufficiently to reduce the incidence of FNHTRs.<sup>40-43</sup>

High-efficiency leukocyte reduction filters for red cells and platelets have been developed that are capable of removing both microaggregate debris and nonaggregated leukocytes.<sup>44</sup> These leukocyte reduction filters can remove 3 or more log (99.9% or more) leukocytes, thereby decreasing the leukocyte content to approximately  $1 \times 10^6$ /unit or less. Despite their efficacy in

leukocyte reduction, the use of bedside leukocyte reduction filters has had variable and sometimes disappointing results in reducing the incidence of FNHTRs to platelet concentrates.<sup>3</sup> This may be the result of causes of FNHTR other than leukocyte antibodies in the transfusion recipient. For example, storage-generated, extracellular cytokines in the component bag that either are not removed or are inadequately removed by means of poststorage filtration are now believed to mediate some reactions. As a result, the practice of prestorage leukocyte reduction is increasingly replacing poststorage leukocyte reduction. Prestorage leukocyte reduction not only removes leukocytes but also removes leukocytes before they have a chance to release cytokines that can accumulate extracellularly in blood component bags during storage. Prestorage leukocyte reduction has also yielded conflicting results on the efficacy of leukocyte reduction to mitigate FNHTRs.<sup>19,20,45</sup>

Prestorage leukocyte reduction of platelet concentrates or RBC units is achieved by use of blood component containers with in-line leukocyte reduction filters in a closed system between the primary collection bag and satellite containers. Prestorage leukocyte-reduced platelets also can be prepared with some automated apheresis instruments equipped with centrifugation chambers designed to minimize leukocyte collection (so-called *process leukocyte reduction*). Because some data indicate that only approximately 15% of patients who experience an FNHTR will have a similar reaction to the next transfusion, some blood banks provide a leukocyte-reduced component (either prestorage or bedside leukocyte reduced) only when a patient has had two or more documented febrile reactions.<sup>46</sup> This practice is cost-effective but has the disadvantage of subjecting some patients to two uncomfortable reactions before a preventive measure is taken.

Poststorage plasma removal from platelet preparations by saline washing techniques is an alternative to prestorage leukocyte reduction for eliminating extracellular cytokines.<sup>14,22</sup> Prestorage leukocyte reduction by means of filtration is a more efficient and cost-effective way to eliminate extracellular leukocyte-derived cytokines while reducing passenger leukocytes. Moreover, in evaluations of plasma removal from platelet concentrates to reduce the risk of FNHTR, this technique still is associated with FNHTR in a relatively large percentage of recipients. Neither leukocyte reduction nor poststorage plasma removal has been effective in eliminating all FNHTRs to platelet transfusions.

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## Allergic Reactions

### Description

An allergic reaction can be classified as an immediate hypersensitivity response consisting of transient localized or generalized urticaria, erythema, and pruritus. More serious allergic reactions can be complicated by hypotension and angioedema of the face and larynx. Allergic reactions can be categorized as those that

have only cutaneous manifestations and usually are mild, resolving soon after administration of antihistamines. If other organ systems—cardiovascular, respiratory, or gastrointestinal—are involved beyond mild hypotension, particularly if the reaction is serious enough to necessitate treatment beyond antihistamines, the reaction would be considered *anaphylactic* or *anaphylactoid* (see later). Allergic and anaphylactic reactions, however, are part of a continuum. Allergic reactions occur during or soon after transfusion of plasma-containing blood components. Atopic individuals—those with other known allergies—appear at greater risk of allergic reactions. A large retrospective review of reported transfusion reactions noted that 17% of all reactions in a 9-year period were allergic and 1% of reactions were very severe.<sup>47</sup> Other papers report allergic reaction rates of approximately 0.1% for red cells and 0.5% for platelet transfusions.<sup>19,45</sup>

### Etiology

Allergic reactions are mediated by recipient immunoglobulin E (IgE) or non-IgE antibodies to proteins or other allergenic soluble substances in the donor plasma. The result of the hypersensitivity reaction is secretion of histamine from mast cells and basophils, which mediates cutaneous reactions by increasing vascular permeability.

Although the source of histamine in allergic reactions is believed in many cases to be the transfusion recipient's mast cells and basophils, it has been hypothesized that histamine generated by leukocytes in stored cellular blood components may play a role. Several studies have shown that histamine accumulates in the plasma portion of platelet concentrates and RBC units with increasing storage time. However, histamine is not synthesized during storage but rather it leaks into the extracellular plasma.<sup>48,49</sup> These data are consistent with the observation that allergic transfusion reactions also are more common with increasing storage time of blood components.<sup>50</sup> Several of the chemokines that accumulate in the plasma portion of platelet concentrates during blood bank storage, such as IL-8, RANTES, and MIP-1 $\alpha$ , can recruit and activate basophils and stimulate histamine release. Therefore, it is theoretically possible that the infusion of storage-generated donor cytokines during transfusion may contribute to the onset of allergic reactions among transfusion recipients. Consistent with this hypothesis, the platelet-derived cytokine, RANTES, is present at higher levels in platelet concentrates that cause allergic reactions.<sup>51</sup>

Allergic (and anaphylactic) reactions have been reported after infusion of antibodies in donor plasma, such as penicillin antibody infused into recipients receiving penicillin or related antibiotics, and after infusion of drugs in donor plasma, such as penicillin infused into recipients already sensitized to penicillin.

### Diagnosis

Urticaria is readily diagnosed clinically by the presence of the cutaneous wheal-and-flare reaction. Because allergic symptoms usually are mild and are not characteristic of hemolytic transfusion reactions, serologic blood bank investigations to rule out

hemolysis usually are unrevealing. Isolated, mild urticarial reactions not accompanied by other signs and symptoms necessitate minimal diagnostic evaluation. If the reaction is severe, has atypical manifestations, or is accompanied by fever (uncharacteristic of allergic reactions), a more elaborate laboratory evaluation to rule out a hemolytic or septic transfusion reaction is indicated. In the diagnosis of an allergic reaction as transfusion-related, it is important to rule out, if possible, urticarial drug reactions that may be circumstantially attributed to transfusions. Careful attention to the timing of onset of urticaria relative to the transfusion may help avoid this confusion. Administration of medications should generally be discouraged in the peritransfusion period to avoid such confusion.

Even mild allergic reactions should be reported to the blood bank. Monitoring allergic reactions and correlating reactions with any newly implemented changes in blood component collection, processing, storage, or filtration are important in detecting new and unexpected causes of reactions. In the care of patients with repeated allergic reactions, notification of the blood bank allows the blood bank medical director to consult on measures to manage or prevent such reactions in the future.

### Treatment

The patient can be treated with a first-generation, H<sub>1</sub>-blocking antihistamine (adults, 25 to 50 mg diphenhydramine intravenously or orally). If the sedating side effects of first-generation antihistamines must be avoided, newer, less-sedating antihistamines are available for oral administration (adults, cetirizine 10 mg orally, loratadine 10 mg orally, or fexofenadine 60 mg orally); however, parenteral antihistamines are preferred in the management of acute reactions because of their more rapid bioavailability. An H<sub>2</sub> blocker, such as cimetidine (adults, 300 mg intravenously) or ranitidine (adults, 50 mg intravenously) may be added to the H<sub>1</sub> blocker to speed resolution of the reaction. Combining H<sub>1</sub> and H<sub>2</sub> antagonists has given better results in treating patients with allergic reactions in nontransfusion settings than has use of an H<sub>1</sub> antagonist alone.<sup>52,53</sup> For reactions characterized by only localized urticaria, such as a few hives, the transfusion can be temporarily discontinued while an antihistamine is administered. The transfusion can be resumed in approximately 30 minutes if the urticaria has cleared and if no further symptoms occur. For patients with generalized urticaria or a more serious allergic reaction accompanied by facial or laryngeal edema or hypotension, the transfusion should be discontinued and the infusion set with any untransfused blood returned to the blood bank. If laryngeal edema causes breathing difficulties or if hypotension is severe, epinephrine [adult dose, 0.2 to 0.5 mL of 1:1000 solution (0.2 to 0.5 mg) subcutaneously] can be administered.

### Prevention

Transfusion recipients often are given routine premedication with an antihistamine such as diphenhydramine in an effort to prevent or reduce the severity of allergic transfusion reactions,

even when they have had no previous reactions. The value of this approach is uncertain, because few patients have allergic reactions. At least two randomized double-blind placebo-controlled studies of premedication using diphenhydramine and acetaminophen have failed to show a benefit of premedication to reduce reactions.<sup>54,55</sup> When premedication is restricted to patients who have had two or more previous allergic reactions, overall reaction rates do not increase. Accordingly, premedication with an antihistamine should probably be reserved for recipients who have had a previous allergic reaction. For patients with repeated reactions not eliminated by premedication with an H<sub>1</sub> blocker alone, a combination of H<sub>1</sub> and H<sub>2</sub> blockers has been shown more effective.<sup>52,53</sup>

Should premedication not prevent repeated allergic transfusion reactions, another option is to reduce the plasma content of transfused blood components. This can be achieved in RBC and platelet preparations with automated saline “washing.”<sup>56,57</sup> However, washing or plasma removal steps generally should be reserved for patients with two or more serious allergic reactions (eg, those that include angioedema or hypotension) that are not prevented with premedication with both H<sub>1</sub> and H<sub>2</sub> blockers, because cell washing is time-consuming and can delay transfusion. Patients with two or more allergic reactions can undergo testing for IgA deficiency, because this is a reported cause of both allergic and anaphylactic reactions.

## Anaphylactic and Anaphylactoid Reactions

### Description

Anaphylactic reactions are serious and potentially life-threatening immediate hypersensitivity reactions to allergens in the plasma of transfused blood components.<sup>58</sup> These reactions can have a rapid onset beginning as early as seconds to minutes after the start of the transfusion, and can occur with small transfused volumes. Anaphylactic reactions are differentiated from other allergic (urticarial) transfusion reactions by their systemic nature and severity. These reactions generally affect multiple organ systems, as evidenced by cutaneous, respiratory, cardiovascular, and gastrointestinal effects. The symptom complex often includes the rapid onset of laryngeal edema and bronchospasm with stridor, wheezing, coughing, and respiratory distress. Other symptoms include generalized urticaria, erythema, tachycardia, hypotension, nausea, vomiting, diarrhea, and cramping abdominal or pelvic pain. Severe reactions can proceed rapidly to shock, syncope, respiratory failure, and death. Fatal anaphylactic reactions are less common than are fatal hemolytic or septic reactions.

### Etiology

Anaphylactic reactions occur when an allergen present in plasma is transfused to a patient who through previous sensitization has an IgE directed against that allergen.<sup>59</sup> Immunoglobulin E is bound by means of Fc receptors to mast cells and basophils. The

binding of allergen to cell-bound IgE results in cross-linking of IgE and Fc receptors. This cross-linking activates the mast cells and basophils to secrete preformed mediators, such as histamine, as well as newly synthesized mediators, such as leukotrienes, prostaglandins, cytokines, and platelet-activating factor (PAF) (Fig 53-2).<sup>60</sup> PAF induces downstream production of nitric oxide (NO) through inducible and possibly constitutively expressed NO synthase.<sup>61</sup> As a potent vasodilator, NO is believed to be the principal compound causing hypotensive and cardiovascular collapse during anaphylaxis, although the exact mechanism remains under debate.

Anaphylactoid reactions are clinically identical to anaphylactic reactions but occur by mechanisms that do not involve IgE. For example, immune complexes involving antibodies other than IgE may result in complement fixation and generation of

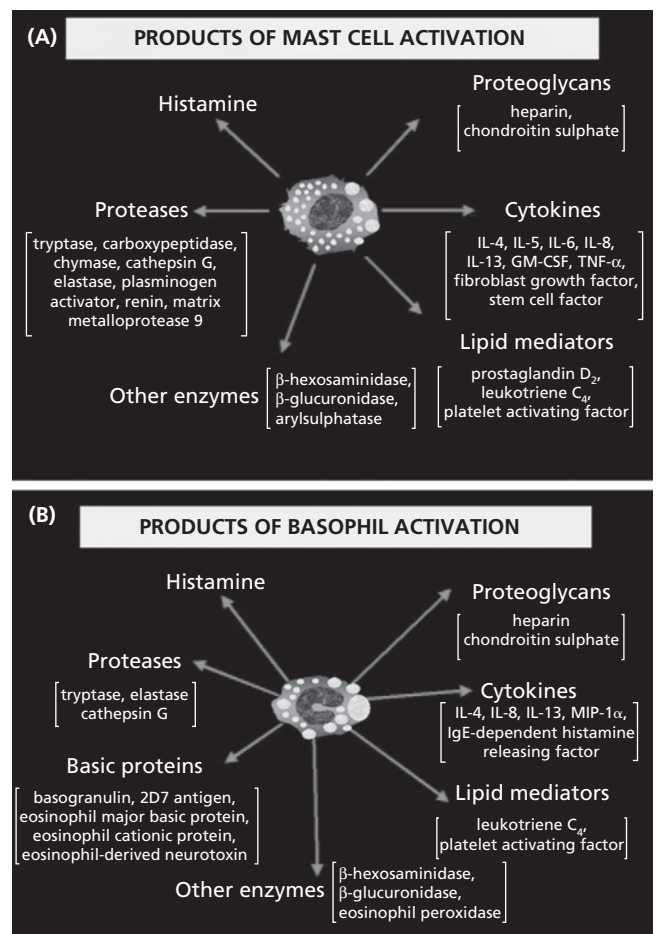


**Figure 53-2.** (A) Mast cell with its activation products. (B) Basophil with its activation products. Note that currently only two products of mast cell activation (histamine and total tryptase) and one product of basophil activation (histamine) can be measured in clinical laboratories as markers of acute anaphylaxis events. Used with permission from Simons FE.<sup>60</sup> IL = interleukin; GM-CSF = granulocyte-macrophage colony-stimulating factor; TNF- $\alpha$  = tumor necrosis factor alpha; MIP = macrophage inflammatory protein.

the anaphylatoxins C3a, C4a, and C5a, which activate basophils and mast cells. Some cytokines secreted by monocytes as part of the inflammatory cascade initiated by non-IgE immune complex formation also can directly activate basophils and mast cells and initiate anaphylactoid reactions. Moreover, IgG4 subclass antibodies can bind to Fc receptors of mast cells and basophils and, in a manner analogous to that of IgE, mediate cellular activation and degranulation after binding of allergen. The term *anaphylactoid* is sometimes used to describe mild or clinically atypical anaphylactic reactions. However, *anaphylactoid* is better used to differentiate the mechanism of the reaction, not its clinical severity or presentation.

The best documented anaphylactoid reactions have resulted from the transfusion of donor plasma containing IgA to IgA-deficient recipients who have produced a class-specific IgG anti-IgA that reacts with all IgA subclasses. Less commonly, patients with normal total IgA levels have a subclass-specific IgA deficiency and may make an anti-IgA of restricted specificity. Although IgA deficiency is relatively common (approximately 1 case among 700 persons), anaphylactoid reactions occur only among some IgA-deficient transfusion recipients, because not all make anti-IgA. Anaphylactic or anaphylactoid reactions have been documented among patients with deficiencies of other plasma proteins, such as complement, von Willebrand factor, and haptoglobin.<sup>62,63</sup> In an analogous manner, these patients produce an antibody to the missing factor that reacts with transfused, plasma-containing blood components. In most anaphylactic or anaphylactoid reactions, however, the allergen is never identified, nor is evidence obtained to differentiate anaphylactic from anaphylactoid mechanisms.

### Diagnosis

Anaphylactic and anaphylactoid reactions are diagnosed from clinical signs and symptoms.<sup>64</sup> The cutaneous signs and symptoms and the often rapid onset of the reaction help differentiate anaphylactic reactions from acute hemolytic and septic transfusion reactions. Serum  $\beta$ -tryptase levels may be measured to confirm an anaphylactic reaction, because it is a marker for mast cell degranulation. However, no laboratory measurement is available in time to meaningfully affect recognition and management of an anaphylactic reaction.

Recipient IgA levels should be measured in a pretransfusion blood specimen to determine if the recipient is IgA-deficient. Although the results of tests for IgA deficiency do not affect diagnosis or management of the reaction at hand, it is important for avoiding future reactions.<sup>58</sup> Testing should be performed on a specimen drawn before transfusion, because IgA deficiency can be masked by any IgA provided by the transfusion. Recipient anti-IgA also can be measured, especially for rare cases in which the anti-IgA is subtype-specific and total IgA levels are within the reference range. Although IgA is the most commonly known allergen in anaphylactoid reactions, in most anaphylactic and anaphylactoid reactions, the offending allergen is not IgA and is never identified.

### Treatment

Anaphylactic and anaphylactoid reactions are managed identically.<sup>64</sup> Severe reactions are true medical emergencies and should be managed by experienced critical care staff, if possible. The patient should be placed in an intensive care unit as soon as it is practical without jeopardizing emergency care. Once anaphylaxis is evident clinically, 1:1000 epinephrine solution (1 mg/mL) should be administered subcutaneously in a dose of 0.2 to 0.5 mL for adults (0.01 mL/kg of body weight for children). The dose may be repeated every 15 to 30 minutes as needed. Intravenous crystalloid or colloid solution should be administered as needed to support the patient's blood pressure. For example, 500 mL to 1 L of normal saline solution can be administered in the first 15 to 30 minutes. Further infusion should be titrated to blood pressure. If the systolic blood pressure is less than 60 mm Hg, intravenous epinephrine in a dose of 1 to 5 mL of a 1:10,000 solution (0.1 mg/mL) for adults and 0.1 mL/kg for children, is administered over 2 to 5 minutes by means of intravenous push. An epinephrine drip (1 to 4  $\mu$ g/minute) may be started, and administration of other pressors, such as dopamine, can be considered. Blood pressure, pulse, and urine output should be monitored. It may be necessary to monitor the effectiveness of fluid replacement and pressor infusion through measurement of central venous pressure.

Respiratory distress is managed with supplemental oxygen. The patient's upper airway may have to be secured with endotracheal intubation if obstruction from laryngeal edema is imminent. Stridor is a sign of laryngeal edema. Endotracheal intubation and mechanical assistance with ventilation are indicated if the PaCO<sub>2</sub> increases to more than 65 mm Hg. When intubation is difficult or impossible because of laryngeal obstruction, cricothyrotomy or tracheostomy is an option. Wheezing caused by obstruction of small bronchi and bronchioles by increased mucus production and smooth muscle contraction can be managed with nebulized albuterol or metaproterenol and intravenous aminophylline.

Urticaria, angioedema, or gastrointestinal distress is managed with an antihistamine (adults, 50 mg diphenhydramine intravenously; children, 1 to 2 mg/kg intravenously). H<sub>2</sub>-blocking antihistamines may be added as an adjunct to H<sub>1</sub> blockers. Glucocorticoids, such as hydrocortisone, 200 mg given intravenously every 6 hours, are also administered because they reduce late-phase inflammatory responses. Glucocorticoids, however, are not expected to be of benefit in the initial management of anaphylaxis because of their delayed onset of action.

### Prevention

Patients with IgA deficiency who have already had an anaphylactic reaction or who are known to have anti-IgA should receive transfusion of RBC and platelet preparations that have been saline-washed with an automated cell washer.<sup>56,58</sup> If plasma transfusion is necessary, only IgA-deficient donors should be used. Patients who have anaphylactic reactions to any other known plasma allergen also should be treated with transfusion

of saline-washed RBCs or platelet preparations. Because anaphylactic reactions can be induced by very small amounts of allergen, washing must be extensive. Washing and saline replacement by means of automated cell washers have been shown generally successful in removing IgA sufficiently to prevent recurrences of anaphylactoid reactions.

If a patient has had one anaphylactic reaction of unknown causation, the next transfusion need not necessarily be performed with washed RBCs or platelets, because the reaction might have been donor-specific. The next transfusion may be administered slowly with vigilance after premedication with both H<sub>1</sub> and H<sub>2</sub> blockers and a glucocorticoid. The patient care team should be prepared to respond to an anaphylactic reaction. The patient ideally should be in a critical care unit with monitoring at the time of transfusion and with a critical care physician and nurses in attendance. Some blood banks with the capability of automated cell washing, nevertheless, may choose to provide saline-washed RBCs and platelet concentrates for future transfusions after a single anaphylactic reaction as a precautionary measure, particularly if the patient is not expected to receive many more transfusions.

## Complications of Massive and Rapid Transfusion

Massive transfusion is defined as the replacement of one blood volume within a 24-hour period. For adults of average size, this is roughly equivalent to 10 units of RBCs with any accompanying crystalloid, colloid, platelet, or plasma infusions. The possible complications include citrate toxicity, electrolyte imbalance (hyperkalemia from transfusion of older RBCs, hypokalemia from citrate toxicity), circulatory overload, and hypothermia. Recipients of massive transfusions are at increased risk of hemolytic transfusion reactions (including ABO incompatibility), FNHTR, and allergic reactions because of the number of units they receive. Reactions can be more severe with massive transfusion because rapid infusion means the implicated unit often has been completely administered before the onset of symptoms. The large number of units transfused in a short time complicates the investigation of transfusion reactions, because each transfused component must be investigated.

The lethal triad of severe trauma consists of hypothermia, acidosis, and coagulopathy. "Damage control" resuscitation methods have been developed to directly address the coagulopathy of trauma. The coagulopathy of trauma develops because of severe injury and is already present when a patient presents for emergency medical care. Trauma coagulopathy is not caused by the resuscitation efforts of emergency medical interventions as traditionally understood.<sup>65</sup> Recent advances in trauma research have shown that early application of damage control resuscitation can greatly improve survival through an application of a 1:1 plasma:red cell ratio from patient presentation. In well-coordinated trauma centers, massive transfusion protocols are under investigation that provide 6 units of plasma, 6 units of RBCs,

6 whole-blood-derived platelet concentrates (or 1 apheresis platelet), and 10 units of cryoprecipitate as a part of a predefined effort to treat the coagulopathy of trauma.<sup>65-67</sup> Aggressive damage control transfusion has been associated with a number of risks including hyperkalemia, and when a walking blood donor program is employed, increased infectious disease transmission.<sup>68,69</sup> Despite these risks, retrospective studies have shown that the application of early, aggressive transfusion support improves overall survival in this severely injured population that would otherwise have very poor prognosis.

Rapid transfusion can also occur during therapeutic apheresis and red cell exchange apheresis (erythrocytapheresis). During apheresis procedures, as many as 10 to 20 units of Fresh Frozen Plasma or 4 to 8 units of RBCs can be transfused over 1.5 to 2 hours. Although any acute transfusion reaction can occur during apheresis-associated transfusion, citrate toxicity in particular is a common but usually mild complication.

### Citrate Toxicity

Rapid blood transfusion can cause a transient decrease in the level of ionized calcium because of the calcium-chelating properties of the citrate anticoagulant in stored blood components.<sup>70</sup> The clinical presentation of citrate-induced symptoms is also termed citrate toxicity. Citrate toxicity can occur whenever large volumes of plasma that contain citrate are transfused, such as during massive transfusion, plasma exchange, or other apheresis procedures.<sup>71,72</sup> Citrate infusion can induce hypocalcemia, hypomagnesemia, and other electrolyte imbalances, and these imbalances are associated with clinical symptoms. Apheresis procedures can produce a unique clinical paradox of urinary calcium excretion in the setting of hypocalcemia.<sup>72,73</sup> Hypocalcemia is a recognized complication of liver transplantation, in which large amounts of plasma are transfused. However, the precise mechanism of hypocalcemia is not well understood and may not be caused entirely by citrated plasma.<sup>74,75</sup>

Citrate ordinarily is rapidly metabolized to bicarbonate in mitochondria-rich tissue, such as liver, skeletal muscle, and kidney.<sup>70</sup> In the routine transfusion of blood components, patients with normal liver function usually tolerate the citrate infusion without significant complications. However, patients with liver or renal failure or parathyroid dysfunction are at greater risk of citrate toxicity when they receive rapid transfusions of plasma or plasma-containing blood components.

Citrate anticoagulates blood by binding divalent cations such as calcium, thus hypocalcemia is a primary symptom. Other divalent cations such as magnesium and zinc can also be bound by citrate, but the contribution of hypomagnesemia to clinical symptoms is less pronounced.<sup>76</sup> During apheresis, citrate is administered as acid-citrate-dextrose formula A (ACD-A) in constant proportion to the whole-blood flow rate. Healthy plateletpheresis donors receive relatively large doses of citrate, and many experience mild symptoms of the citrate effect, but the symptoms usually do not progress because of the short duration of the procedure. Donors of peripheral blood stem cells (PBSCs),

however, receive smaller doses of citrate per unit of time but usually experience more severe citrate toxicity because of the longer duration of the procedure. Paresthesia caused by transient hypocalcemia is common in apheresis. It typically occurs after the initial infusion of the priming solution (if citrate is used) or later as apheresis progresses. Apheresis practitioners should be aware that peripheral paresthesia caused by hypocalcemia can be masked in patients with a preexisting neuropathy as a result of chemotherapy (vincristine) or as part of a neurologic condition.

Citrate toxicity is recognized clinically because of the signs and symptoms of hypocalcemia. It can be confirmed by measuring the plasma ionized calcium level in the patient. Symptoms of hypocalcemia include peripheral and perioral paresthesia (Chvostek and Trousseau signs can occasionally be elucidated), muscle spasm, cramping, nausea, vomiting, cardiac arrhythmia, bradycardia, hypotension, and if severe, tetany. An electrocardiogram (ECG) can show prolongation of the QT interval with hypocalcemia, but the relation is not linear with the ionized calcium level, and ECG findings are an unreliable guide to calcium therapy.

Mild citrate toxicity during transfusion or apheresis is managed or prevented in part by means of slowing the rate of transfusion or reinfusion. When slowing the infusion rate is impossible or ineffective and the patient has signs and symptoms of hypocalcemia, calcium supplementation is indicated. The best guide to determining a need for calcium supplementation is measurement of the patient's ionized calcium levels, if results can be obtained rapidly. Calcium replacement during apheresis should generally be given when a patient has symptoms, when the patient's clinical condition may exacerbate citrate effects, or when prolonged large-volume leukapheresis is expected to cause citrate toxicity. Infusion of calcium itself, however, is associated with development of ventricular arrhythmia and even cardiac arrest. Therefore, intravenous calcium replacement for the management or prophylaxis of apheresis-induced citrate toxicity should be administered only by experienced apheresis staff. Under no circumstances should calcium be added directly to a unit of blood, because it causes clots to form in the bag.

Citrate toxicity during apheresis is related to the citrate concentration of the reinfused blood or colloid solution, the infusion rate, the blood volume of the patient, and the total time over which the citrate is infused.<sup>77</sup> It is difficult to establish a definitive safe rate of citrate infusion because of the large number of variables involved. However, citrate dosages of up to 1 mg/kg/minute given during plateletpheresis usually are well tolerated. A safe rate of calcium replacement for controlling citrate toxicity during PBSC apheresis is 0.5 to 0.6 mg of calcium ion for every 1.0 mL of infused ACD-A.<sup>78,79</sup> These dosages have been successful for prophylaxis against citrate toxicity during large-volume leukapheresis. To avoid excessive volume during PBSC apheresis, administration of a concentrated calcium solution (calcium chloride or calcium gluconate) is appropriate. Care must be taken to coordinate the calcium infusion with whole blood flow during the apheresis procedure to avoid the potential for catheter

thrombosis. Calcium administration should be halted soon after interruptions in whole blood flow.

### Electrolyte Disorders

Because of inhibition of sodium-potassium-adenosine triphosphatase in red cell membranes by the cold storage temperature of RBC units, extracellular potassium accumulates with increasing blood bank storage times. Extracellular potassium increases at the rate of approximately 1 mEq/day during the first 3 weeks of RBC storage in citrate-phosphate-dextrose-adenine-1 (CPDA-1).<sup>80</sup> Potassium levels in additive solution-1 (Adsol) units are markedly higher on Day 7 of storage (17 mmol/L) than on Day 0 (1.6 mmol/L). The increase is even greater by Day 42 (46 mmol/L).<sup>81</sup>

Hyperkalemia resulting from massive transfusion of older RBC units with an elevated amount of extracellular potassium can cause significant cardiac complications and possibly death in some patients.<sup>68</sup> Acidosis can contribute to hyperkalemia and severely injured patients presenting with a potassium level greater than 4 mmol/L are at increased risk. Other patients at risk of hyperkalemic complications are neonates and those with renal failure. The diagnosis of hyperkalemia is made by means of measurement of potassium in the serum and observation of ECG changes, which include peaked T waves, prolongation of the PR interval, and ventricular arrhythmia.<sup>82</sup> In neonatal transfusions, hyperkalemia can be avoided by use of fresh RBC units (less than 7 days old) or older units that have been saline-washed to remove the extracellular fraction containing the potassium.<sup>83,84</sup> However, transfusion of older RBC units does not place neonates at risk of hyperkalemia if small-volume transfusions (10 to 15 mL/kg) are given slowly.<sup>85</sup>

Hypokalemia can also develop during massive transfusion or large volume apheresis.<sup>72</sup> As the anticoagulant citrate in blood components is metabolized to bicarbonate, the blood can become alkalotic, producing hypokalemia. The degree of hypokalemia may be sufficient to necessitate infusion of potassium if symptoms develop. However, the use of newer RBC additive solutions such as Adsol has helped to decrease the effect of hypokalemia. RBC units are plasma-reduced before the addition of additive solution, which itself contains no additional citrate. Therefore, most of the citrated plasma is removed from additive solution RBC units during production. Animal studies have shown fewer physiologic aberrations during massive transfusion with Adsol RBCs than with CPDA-1 units.<sup>86</sup> The complications of hypokalemia, therefore, are more likely when large numbers of units of plasma rather than RBC units are transfused.

### Hypothermia

Hypothermia, defined as a core body temperature of less than 35°C, may be caused by rapid infusion of large quantities of cold (1°C to 10°C) blood or RBC units. Hypothermia during massive transfusion has been shown to induce cardiac arrhythmia and arrest.<sup>87</sup> Even smaller quantities of cold blood can be cardiotoxic if transfused into central venous lines, because the newly infused cold blood can reach the heart before sufficient warming has



occurred. Data published in the early 1960s showed that massive transfusion at a rate of approximately 1 unit every 5 to 10 minutes was sufficient to lower the temperature of an esophageal probe behind the right atrium to nearly 30°C.<sup>87</sup> The resulting decrease in sinoatrial node temperature was associated with the development of ventricular fibrillation.

For most routine transfusions given at a standard rate of administration, blood does not have to be warmed.<sup>88</sup> The patient may experience minor chills, but this is easily remedied by warming the patient, as with extra blankets. Transfusion of cold RBC units through central venous lines, however, should be avoided. Indications for warming blood include rapid transfusion, which are generally considered to be more than 50 mL/kg/hour for an adult and more than 15 mL/kg per hour for a child, and exchange transfusion for infants. Because blood warming during certain massive transfusions sometimes delays infusion and impedes resuscitation, it is not always practical. Warming blood for transfusion in the treatment of patients with cold agglutinin disease has a theoretic basis but is debatable because supportive outcome data are lacking.

If blood has to be warmed, an approved warming device should be used and the temperature must be kept below a point where hemolysis occurs.<sup>88</sup> Infusion of thermally injured cells can induce disseminated intravascular coagulation and shock. Heating blood with a device other than an approved blood-warming device, such as a commercial microwave oven, is unacceptable. Blood that has been warmed but not used should not be reissued for another patient because of the increased risk of bacterial proliferation at warmer temperatures.

The maximum flow rate that can be achieved with commercially available blood warmers is 850 mL/minute; however, most can provide a rate of only 150 mL/minute. Most recent research has focused on comparisons between commercial warming devices and the comparison studies usually evaluate warming capability in the rapid transfusion setting.<sup>89</sup> Most of the available federally approved blood warming devices safely warm blood and other intravenous fluids across a range of flow rates. However, while little recent data exist correlating the clinical benefits of warming blood, many emergency centers and trauma services use such devices routinely without incident.

An alternative to using mechanical blood warmers that circumvents such flow limitations is rapid admixture with warm or hot saline solution immediately before transfusion.<sup>88,90</sup> This technique immediately warms a unit of RBCs, yet does not cause significant hemolysis. However, it necessitates that warmed saline solution be available at all times in trauma care and requires attention to technique to avoid the direct infusion of hot saline solution into the patient.

### Reactions Attributed to Microaggregate Debris

Microaggregate debris ranges in diameter from 20 to 120 microns and consists of nonviable platelets, white cells, and strands of fibrin that form in blood during storage.<sup>91,92</sup> Because of their size, microaggregates are not removed from transfused blood with the standard 170- to 260-micron screen filters. A variety of

adverse events have been attributed to the presence of microaggregate debris after large-volume and massive transfusion.

Studies in the 1960s showed that patients undergoing open-heart surgery with cardiopulmonary bypass experienced postperfusion syndrome during the postoperative period. This symptom complex consisted of cerebral and renal dysfunction attributed in part to occlusion of end-organ capillaries with microaggregate debris. Cotton wool (Swank) microaggregate blood filters capable of retaining particles or debris with a size of 40 microns or more appeared to eliminate many of these reactions. During the Vietnam War, some soldiers who underwent massive transfusion experienced respiratory distress syndrome (shock lung). At autopsy, the cause was presumed to be the material found in soldiers' lungs that was positive on a periodic acid-Schiff (PAS) test. Because microaggregate debris stains PAS-positive, this was taken at the time as evidence of the pathologic nature of microaggregate debris.

During the 1970s and 1980s, studies were undertaken to determine whether removal of microaggregate debris from blood was clinically significant.<sup>91</sup> Several studies showed that microaggregate filtration of up to 6 units of blood during either hip or cardiac surgery provided no benefit. Collins et al<sup>93</sup> concluded that the underlying clinical condition rather than the infusion of the microaggregate debris in blood led to the development of the respiratory distress syndrome reported earlier among patients undergoing massive transfusions. Microaggregate blood filters today are used mostly in conjunction with cardiopulmonary bypass pumps and are only rarely used otherwise. With the widespread adoption of leukocyte reduction filtration, routine leukocyte-reduced red cell transfusions are no longer complicated by microaggregate debris because these filters can remove not only leukocytes, but also the larger microaggregate particles. Current studies are investigating the utility of washing stored blood to remove microaggregate debris that accumulates during storage.<sup>94</sup>

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### Circulatory Overload

Hypervolemia, termed transfusion-associated circulatory overload, is a possible consequence of transfusion in the care of patients with cardiac insufficiency, renal impairment, or already expanded blood volumes, such as patients with chronic anemia. Moreover, patients with restricted blood volumes (such as infants and small children) are at risk of TACO if transfused blood is not reduced to an amount proportional to body mass and intravascular blood volume. The risk of TACO increases with rapid infusion. Circulatory overload increases central venous pressure, causes congestion of the pulmonary vasculature, and decreases lung compliance, manifesting as dyspnea, tachycardia, acute hypertension, and in the extreme, pulmonary edema and left- or right-sided heart failure. Other signs and symptoms of circulatory overload include tachypnea, dry cough, chest or throat tightness, jugular venous distention, and pulmonary rales. Laboratory measurements of circulatory overload include PaO<sub>2</sub>, atrial natriuretic peptide and B-type natriuretic peptide.<sup>95,96</sup> The

latter has a reported 81% sensitivity for detecting circulatory overload in the appropriate clinical setting. Diagnosing TACO can be difficult and confounded by other concomitant pathology. There is no accepted clinical definition of TACO and the symptoms of TACO can overlap significantly with other transfusion reactions such as TRALI (Table 53-1).<sup>97,98</sup> Some rapid methods of differentiating the overlapping symptoms include blood pressure, response to diuretic therapy, white cell count, and heart auscultation for an S3 (third heart sound).

If symptoms of overload appear, the transfusion should be stopped, and intravascular volume reduction through diuresis should be instituted as needed (eg, administration of 40 mg furosemide intravenously). The patient should be placed in an upright (reverse Trendelenburg) position, if possible, with supplemental oxygen as necessary. Phlebotomy may be considered for severe volume overload, although this is not usually prudent in the setting of anemia or hypoxemia.

Rapid transfusion of any blood component into a patient who is not actively hemorrhaging produces no benefit and can cause harm. As a general guide, infusion should be at a rate not to exceed 2 to 4 mL/kg/hour, and the rate should be lower (~1 mL/kg/hour) for patients at high risk of circulatory overload.<sup>99</sup> In neonates, a slower blood infusion rate increases the hematocrit and decreases cardiac demand without affecting pulmonary artery pressure. More rapid infusion rates are associated with decreased lung compliance and increased pulmonary airflow resistance.<sup>100,101</sup>

For patients with volume overload caused by medical reasons existing before transfusion, furosemide can be given prophylactically, and transfusion should proceed slowly. The rate of transfusion can be even further slowed, if necessary, by dividing a unit of RBCs or another component into smaller

aliquots and transfusing a portion at a time over as much as 4 hours, the maximum allowable time a blood component should be kept outside blood-bank-monitored storage. For RBCs and thawed plasma, the unused portion can be stored in the blood bank at 1°C to 6°C for up to 24 hours while the initial aliquot is administered. Platelet aliquots can be sampled from a single apheresis platelet unit, and with this practice, donor exposures can be minimized. It is important that transfusion of all or part of a blood component be completed within 4 hours and that any unused portion be stored under regulated blood bank conditions because of concerns about increased risk of bacterial contamination during improper storage. RBC units, apheresis platelets, and whole-blood-derived platelet pools can be further concentrated by means of centrifugation and plasma removal, if other measures to prevent volume overload are inadequate.

**Toxic Reactions Resulting from Blood Manufacture or Processing**

**Hypotensive Reactions**

Hypotension has been reported among patients receiving bedside, leukocyte-reduced platelets who are also medicated with angiotensin-converting enzyme (ACE) inhibitors.<sup>102</sup> These reactions appear to be caused by generation of bradykinin in transfused blood just as it is being passed through negatively charged leukocyte reduction filters. The mechanism is believed to involve the formation of activated Factor XIIa when Factor XII, a contact factor, is exposed to the negatively charged filter surface. The filter surface can mimic exposed, negatively charged subendothelium, which is the natural activating stimulus for the contact factors

**Table 53-1.** Features in TRALI and TACO

| Feature               | TRALI                                                                                      | TACO                                                                                                               |
|-----------------------|--------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------|
| Body temperature      | Fever can be present                                                                       | Unchanged                                                                                                          |
| Blood pressure        | Hypotension                                                                                | Hypertension                                                                                                       |
| Respiratory symptoms  | Acute dyspnea                                                                              | Acute dyspnea                                                                                                      |
| Neck veins            | Unchanged                                                                                  | Can be distended                                                                                                   |
| Auscultation          | Rales                                                                                      | Rales, S3 may be present                                                                                           |
| Chest radiograph      | Diffuse, bilateral infiltrates                                                             | Diffuse, bilateral infiltrates                                                                                     |
| Ejection fraction     | Normal, decreased                                                                          | Decreased                                                                                                          |
| PA occlusion pressure | 18 mmHg or less                                                                            | Greater than 18 mmHg                                                                                               |
| Pulmonary edema fluid | Exudate                                                                                    | Transudate                                                                                                         |
| Fluid balance         | Positive, even, negative                                                                   | Positive                                                                                                           |
| Response to diuretic  | Minimal                                                                                    | Significant                                                                                                        |
| White count           | Transient leukopenia                                                                       | Unchanged                                                                                                          |
| BNP                   | <200 pg/mL                                                                                 | >1200 pg/mL                                                                                                        |
| Leukocyte antibodies  | Donor leukocyte antibodies present, crossmatch incompatibility between donor and recipient | Donor leukocyte antibodies may or may not be present, positive results can suggest TRALI even with true TACO cases |

The typical patterns that would be expected for cases of transfusion-related acute lung injury (TRALI) or transfusion-associated circulatory overload (TACO) are represented. A given case of TRALI or TACO may lack some of the typical features. Also, a case of TRALI may have some features suggesting TACO or vice versa, and TRALI and TACO can be present together. The best strategy is to develop a full clinical profile of the case using the feature list above, and determine which diagnosis is most supported.

BNP = brain natriuretic peptide; PA = pulmonary artery.

of the intrinsic coagulation pathway after blood vessel damage *in vivo*. Factor XIIa converts prekallikrein to kallikrein, which cleaves high-molecular-weight kininogen to form bradykinin. The biologic activity of the infused bradykinin is prolonged in transfusion recipients who are also receiving ACE inhibitors (eg, captopril, enalapril), which inhibit kininase II, the enzyme that breaks down bradykinin. The combination of bradykinin generation just as the blood is being infused with inhibition of the transfusion recipient's ability to break down bradykinin produces prolonged bradykinin activity conducive to hypotensive reactions.

These reactions are less likely with use of prestorage leukocyte-reduced blood components, because the bradykinin is broken down rapidly in the component bag during storage before transfusion. Although hypotensive reactions have been reported more frequently with negatively charged bedside leukocyte reduction filters, they also have been rarely reported with positively charged filters. This can be explained in part by the possibility that patients taking ACE inhibitors may be more prone to hypotensive reactions in general because of their relative inability to rapidly break down bradykinin generated *in vivo* by any allergic mechanism. Hypotensive reactions to bedside leukocyte reduction among patients taking ACE inhibitors can be prevented by use of prestorage leukocyte-reduced blood components or by means of temporary discontinuation of ACE inhibitor treatment.

Apheresis procedures are also associated with hypotensive reactions and the literature has described hypotensive reactions in both adult and pediatric apheresis patients.<sup>103,104</sup> Apheresis may contribute to hypotensive reactions through several mechanisms including the potentiation of bradykinin-mediated effects by albumin and secondary to hypocalcemia.<sup>104,105</sup> Data suggest that calcium infusions can mitigate some atypical apheresis reactions, while withholding ACE inhibitor medications 24 to 48 hours before apheresis may also contribute to lessening reactions.

### Ocular Reaction to Leukocyte-Reduced Blood Components: Red Eye Syndrome

Some patients receiving transfusions of RBCs prestorage leukocyte reduced with a specific filtration system (LeukoNet Prestorage Leukocyte Reduction Filtration System, HemaSure, Marlborough, MA) sustained bilateral conjunctival erythema (red eye syndrome).<sup>106</sup> The conjunctival erythema occurred within 24 hours of transfusion. Resolution occurred spontaneously within 2 to 21 days with a median duration of 5 days. The implicated prestorage leukocyte reduction system has been discontinued, and red eye syndrome has not been reported with other leukocyte reduction filters.<sup>107</sup> The red eye symptoms are hypothesized to be an allergic or toxic reaction to cellulose acetate derivatives that leached from the filter membrane.

### Plasticizer Toxicity

Plasticizers are chemicals used to make rigid polyvinyl chloride plastics more malleable. The traditional plasticizer for blood storage bags is di(2-ethylhexyl)phthalate (DEHP), which leaches over time from the plastic into the blood and blood components

with increasing exposure. The DEHP metabolite, mono(2-ethylhexyl)phthalate (MEHP), also accumulates during storage.<sup>108</sup> Infusion of blood that contains DEHP results in deposition of DEHP in various tissues; the greatest accumulation is in body fat. Results of some studies with animals have suggested that DEHP is toxic and may even be carcinogenic in large quantities.<sup>109</sup> Other studies with animals have shown that MEHP is associated with formation of peroxisomes, indicating tissue alteration and toxicity. Although there have been no reports of transfusion-related plasticizer toxicity among humans, results of some *in-vitro* experiments suggest that high concentrations of MEHP have a negative inotropic effect and can cause irregular contractions in isolated human myocardial cells. Some clinical data have described the production of antiplasticizer IgE in transfusion recipients and the incorporation of plasticizer into red cells during storage.<sup>110</sup> Despite the possible adverse effects of DEHP plasticizers, other data indicate that these substances stabilize red cell membranes and improve the morphologic features of platelets during storage.<sup>111,112</sup> No good evidence exists, however, of actual improvement in posttransfusion outcomes as the result of these effects.

Formulations for plastic blood bags are being developed with plasticizers other than DEHP that have a decreased capacity to leach into plasma. For example, one polyvinyl-chloride-based material is made with plasticizer butyryl tri-*n*-hexyl citrate (BTHC). Although BTHC also leaches into blood components, it does so at a significantly slower rate than does DEHP. It also provides an antihemolytic effect similar to that of DEHP.<sup>113</sup> Studies have shown this citrate-based plasticizer is suitable for storage of both RBCs and platelets.<sup>114</sup> Given the concerns over the risk of DEHP toxicity, other plasticizers and storage materials continue to be sought in an attempt to replace traditional storage materials. The possible human toxicity of DEHP continues to be evaluated.<sup>115-117</sup> (See also Chapter 4.)

### Dimethyl Sulfoxide Toxicity During Infusion of Cryopreserved Progenitor Cells

Dimethyl sulfoxide is a versatile solvent that has been used as the principal cryopreservative for mononuclear cells since the 1950s. It is widely used as a cryopreservative for marrow and PBSCs used in human hematopoietic progenitor cell transplantation. Despite this, DMSO is not approved by the Food and Drug Administration as a pharmacologic agent for intravenous administration, and guidelines for intravenous administration are obscure. Toxicologic studies, however, have established the general safety of intravenous DMSO infusion.<sup>118,119</sup> The metabolism of DMSO yields a characteristic harmless odor, described as a malodorous garlic or sulfur-like smell. Because of the exceptional solvent properties of the compound, DMSO is distributed throughout all tissues after administration. The two metabolites of DMSO are dimethyl sulf dioxide and dimethyl sulfide (DMS). Dimethyl sulf dioxide is an odorless compound excreted by the kidney, and DMS is excreted through the lungs and through other tissues and contributes to the characteristic odor.

The clinical toxicity of DMSO in marrow transplantation has been studied. Anaphylactoid symptoms attributable to the release

of histamine and other mediators are common. Other toxic clinical signs and symptoms include hemolysis with hemoglobinuria, hyperosmolality, increased serum transaminase values, nausea, vomiting, abdominal cramping, fever, chills, tachypnea, cough, diarrhea, flushing, and headache.<sup>120,121</sup> Patients who have been conditioned with chemotherapy or who have smaller body mass (<70 kg), seem more likely to experience nausea and vomiting after infusion of DMSO-preserved cells. Cardiovascular toxicities include decreased heart rate and bradycardia, occasionally increased heart rate and tachycardia, ectopic heartbeat, heart block, hypotension, hypertension, and other lesser blood pressure changes.<sup>120,122</sup> Some studies, however, raise the question whether there is any significant cardiovascular toxicity of DMSO.<sup>123</sup> It is possible that some adverse effects attributed to DMSO may be caused by the cellular infusion itself.

The mechanism of the clinical toxicities associated with DMSO infusion has not been well established. Histamine receptor binding of DMSO, histamine release, direct vagal tonic effects, cold thermal vagal responses, and renal failure secondary to hemolysis explain many of the symptoms observed during cryopreserved cellular infusion.<sup>120,124</sup> Increases in thrombin-antithrombin complex,  $\beta$ -thromboglobulin, platelet factor 4, and von Willebrand factor caused by DMSO have been described.

Several measures can be taken to prevent or reduce DMSO toxicity. Antihistamine prophylaxis is recommended routinely before any administration of DMSO. Intravenous DMSO should be given as a 10% to 40% solution to avoid local irritation. The recommended maximum daily dose of DMSO is 1 g/kg/day. Slowing an infusion containing DMSO or increasing the time between infusions of multiple aliquots greatly diminishes DMSO-related toxicity, which appears to be a dose-dependent but short-lived response. However, because DMSO is toxic to thawed mononuclear cells, hematopoietic progenitor cells can tolerate exposure to 10% DMSO for only as long as 1 hour.<sup>125</sup> This limits how much the infusion rate can be slowed. Antiemetics and sedatives can help to ameliorate symptoms, and cellular products can be carefully washed before infusion to remove DMSO and other substances.

## Reactions in Special Transfusion Settings

### Granulocyte Transfusion Reactions

Granulocyte transfusions remain in use as a treatment option for neutropenic patients because of improved granulocyte collection yields after donor treatment with steroids and granulocyte colony-stimulating factor (G-CSF). Febrile nonhemolytic transfusion reactions after granulocyte transfusion are common. Severe reactions can be accompanied by pulmonary complications (eg, TRALI, dyspnea, pulmonary infiltrates, and hypoxia), hypotension, and even cardiovascular collapse.<sup>126</sup> In a recent study of dexamethasone- and G-CSF-stimulated granulocyte transfusions, 37% of patients (7% of transfusions) experienced chills, 32% of patients (7% of transfusions) experienced a fever, and 11% of patients (2% of transfusions) experienced hives

or itching during a course of therapy.<sup>127</sup> Oxygen desaturation of greater than 3% occurred in 7% of transfusions, and severe desaturation of greater than 6% occurred in three of 11 patients experiencing oxygen desaturation. In addition, granulocyte transfusions carry further risk of leukocyte alloimmunization and cytomegalovirus infection.<sup>128</sup>

Concurrent administration of amphotericin B and granulocytes has been linked to severe pulmonary reactions, although the association has not been confirmed and remains in doubt. Nevertheless, it is prudent to separate amphotericin B administration and granulocyte therapy by at least 6 hours to avoid confusion about the cause of a severe reaction, which can occur with either of these reaction-prone treatments. Because of the relatively high rate and severity of febrile, pulmonary, and allergic reactions, it is prudent to give premedication with acetaminophen and diphenhydramine to recipients of granulocyte transfusions. Hydrocortisone may be added as premedication in the treatment of patients with severe reactions who otherwise cannot tolerate granulocyte transfusion, although the immunosuppressive effects of this agent are unwelcome among patients who need granulocyte transfusions to fight serious and life-threatening infections. Granulocyte concentrates should be transfused slowly.

### Autologous Transfusion Reactions

A variety of reactions to autologous blood occur despite the complete compatibility. In a study involving 596 hospitals, the rate of reported FNHTRs to autologous blood was 0.12% and the rate of allergic reactions was 0.01% per transfused unit.<sup>129</sup> Such rates are approximately fivefold to 10-fold lower than those reported for allogeneic units. The cause of autologous transfusion reactions has not been clearly established. Mechanisms in many cases presumably are the same as for allogeneic transfusions. For example, autologous units can be contaminated with bacteria as can allogeneic units, and contamination leads to febrile reactions or septic complications.<sup>130</sup> Because autologous donors are patients, not healthy volunteers, they may have various medical problems that put them at increased risk of bacteremia.

Accumulation of inflammatory mediators in blood component bags during storage, released from passenger leukocytes or platelets, may result in the infusion of pyrogenic substances. Autologous leukocytes, rather than allogeneic leukocytes, can also generate endogenous pyrogens. Allergic reactions may be provoked by histamine generation during storage of blood components or by chemicals leached from blood storage containers or filters. Moreover, autologous transfusions may contribute to volume overload and hypervolemic reactions through mechanisms identical to allogeneic transfusions.

## Summary

A variety of acute, nonhemolytic and noninfectious reactions are reported after transfusion (Table 53-2). Many of these reactions

**Table 53-2.** Transfusion Reaction Summary

| Type                                                      | Cause                                                                                                                                                                                                  | Signs and Symptoms                                                                                                                                                                                | Treatment                                                                                                                                                                                                                                                                  | Prevention                                                                                                                                                                                                                                           |
|-----------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Febrile, nonhemolytic</i>                              | Recipient antibodies against leukocytes or platelets in donor blood components; cytokines in plasma or supernatant portion of stored components; undetected bacterial contamination of blood component | Chills, fever ( $>1^{\circ}\text{C}$ increase in body temperature); rigors in severe reactions                                                                                                    | Stop transfusion, notify physician and blood bank, maintain IV line, monitor vital signs; physician may order acetaminophen                                                                                                                                                | Premedicate with acetaminophen (or glucocorticoid for refractory cases); give leukocyte-reduced RBCs                                                                                                                                                 |
| <i>Allergic</i>                                           | Allergen is a soluble substance in donor plasma                                                                                                                                                        | Localized or generalized urticaria, erythema and pruritus; if severe, may have laryngeal or facial angioedema, and hypotension                                                                    | Hold transfusion, notify physician, monitor vital signs; physician may order antihistamines or restart of transfusion if mild urticaria clears and no other symptoms in 30 minutes                                                                                         | Premedicate with $\text{H}_1$ blocking antihistamine; add $\text{H}_2$ blocker or glucocorticoid for refractory cases; consider washed RBCs and platelets for repeated or severe reactions                                                           |
| <i>Anaphylactic or anaphylactoid</i>                      | Recipient antibodies to a soluble substance in donor plasma; infusion of plasma with IgA into IgA-deficient recipient with anti-IgA                                                                    | Urticaria, flushing, angioedema, stridor, wheezing, tachycardia, hypotension, shock, abdominal pain, diarrhea, pelvic pain                                                                        | Stop transfusion; maintain IV line; notify physician and blood bank, monitor vital signs; physician may order antihistamines, epinephrine, oxygen, IV crystalloid, or glucocorticoids                                                                                      | Premedicate with antihistamines and glucocorticoid; transfuse washed RBCs and platelets for recurrent reactions; use IgA-deficient donors or washed RBCs and platelets for sensitized patients with IgA deficiency                                   |
| <i>Transfusion-associated circulatory overload (TACO)</i> | Blood volume too large or infusion too fast for compromised cardiovascular system                                                                                                                      | Dyspnea, orthopnea, systolic hypertension, headache, peripheral edema, coughing, cyanosis                                                                                                         | Slow or stop transfusion; keep IV line open; notify physician; monitor vital signs and input and output; physician may order diuretics and oxygen                                                                                                                          | Transfuse slowly; use split units; consider premedication with diuretics; carefully monitor aged, debilitated, or cardiac patients                                                                                                                   |
| <i>Hypothermia</i>                                        | Core body temperature $<35^{\circ}\text{C}$ caused by rapid infusion of cold blood products, such as RBCs, FFP, cryoprecipitate                                                                        | Decreased body temperature, chills, cardiac arrhythmia (ventricular fibrillation)                                                                                                                 | Slow or stop transfusion; use an approved blood warmer, blankets, and other patient warming techniques (warm lavages, lamps)                                                                                                                                               | Transfuse slowly, use an approved blood warmer                                                                                                                                                                                                       |
| <i>Citrate toxicity</i>                                   | Excessive infusion of citrate during apheresis procedure or massive or rapid transfusion; patients with liver failure are at increased risk                                                            | Perioral or peripheral paresthesia, tingling, buzzing, teeth chattering, bed or chair moving, cramps, nausea, vomiting, arrhythmia, bradycardia, hypotension, prolongation of QT interval, tetany | Slow or stop transfusion; slow or stop apheresis procedure; give IV calcium chloride or gluconate (for PBSC apheresis: 0.5 mg $\text{Ca}^{2+}$ /1.0 mL ACD-A), or check ionized $\text{Ca}^{2+}$ and dose per results; monitor relief of symptoms; consider hypomagnesemia | More likely in lightweight patients ( $<70$ kg) and patients with liver dysfunction, renal failure, or less skeletal muscle; observe patients closely for any symptoms, give IV calcium (for PBSC apheresis: 0.5 mg $\text{Ca}^{2+}$ /1.0 mL ACD-A). |

(Continued)

Table 53-2. (Continued)

| Type                        | Cause                                                                                                                                                                                                                                                                     | Signs and Symptoms                                                                                                                                                                                   | Treatment                                                                                                                                | Prevention                                                                                                                                                                                                                                                                     |
|-----------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Electrolyte disorder</i> | Hyperkalemia: transfusion of older blood components or massive transfusion of RBCs                                                                                                                                                                                        | Hyperkalemia: cardiac arrhythmia, ECG changes—peaked T waves, prolongation of PR interval (if severe, flat or lost P wave), widened QRS, ventricular arrhythmia                                      | Hyperkalemia: give calcium to protect against cardiac effect, alkalinize blood, D50 plus insulin, sodium polystyrene sulfonate; dialysis | Hyperkalemia: give fresh products (<7 days old), or washed products                                                                                                                                                                                                            |
|                             | Hypokalemia: massive or rapid transfusion of citrate and metabolic alkalosis                                                                                                                                                                                              | Hypokalemia: cardiac arrhythmia, ECG changes—ST depression, U waves                                                                                                                                  | Hypokalemia: give potassium                                                                                                              | Hypokalemia: give Adsol-preserved RBCs (not Nutricel).                                                                                                                                                                                                                         |
| <i>Hypotensive</i>          | Bradykinin generation with use of negatively charged bedside leukocyte reduction filters in patients taking angiotension-converting enzyme (ACE) inhibitors; apheresis procedures using albumin, especially in patient's taking ACE inhibitors; see also citrate toxicity | Hypotension; sometimes also flushing, respiratory distress, nausea, abdominal pain, and loss of consciousness                                                                                        | Stop transfusion, notify physician and blood bank; support blood pressure.                                                               | Avoid use of bedside leukocyte reduction filters in patients taking ACE inhibitors; use prestorage leukocyte-reduced components or discontinue ACE inhibitor before transfusion or apheresis procedure; if during apheresis, correct electrolyte disorder such as hypocalcemia |
| <i>DMSO toxicity</i>        | Cryopreservative for bone marrow, PBSCs, donor lymphocyte infusions, or any frozen cellular component; toxicity with DMSO >1.0 g/kg/day                                                                                                                                   | Flushing, nausea, vomiting, abdominal cramping, throat tightness and cough, hypotension, hypertension, arrhythmia, fever, chills, headache, hemoglobinuria, hyperosmolality, increased liver enzymes | Antihistamines; antiemetics; slow or stop the infusion; supportive care; wait between infusions for symptoms to clear                    | Antihistamines; washed or plasma or volume depleted cellular infusions; antiemetics                                                                                                                                                                                            |

IV = intravenous; RBCs = Red Blood Cells; FFP = Fresh Frozen Plasma; PBSC = peripheral blood stem cell; ACD-A = acid-citrate-dextrose-adenine; D50 = dextrose 50% in water; ECG = electrocardiogram; DMSO = dimethyl sulfoxide.

have an immune basis and represent inflammatory or allergic responses to infused cells (eg, many FNHTRs) or plasma (eg, some FNHTRs, allergic, and anaphylactic reactions). Although urgent transfusion can be lifesaving, it is important to recognize that a large volume of blood components given too quickly can itself have adverse chemical or physical effects, such as hypothermia, hyperkalemia, hypokalemia, hypocalcemia, and circulatory overload. Some reactions also are caused by unintended consequences of blood storage conditions or processing, such as generation of bradykinin by the contact of blood with some filter surfaces, leaching of toxic chemicals from filters or containers, and use of the chemical DMSO during hematopoietic progenitor cell preservation. It is important that these reactions and toxicities be recognized rapidly by the patient care team and blood bank personnel so that appropriate treatment and preventive measures can be instituted quickly. Care providers who administer transfusions must recognize that some symptoms of transfusion reactions, such as fever, are nonspecific and may be early manifestations of potentially life-threatening reactions, such as hemolysis or sepsis (Table 53-3). For that reason, the guiding rule regarding most transfusion reactions is to err on the side of conservatism and stop the transfusion immediately. Transfusion of a blood component that causes a reaction before complete infusion should not be restarted, with the possible exception of mild urticarial reactions. Several strategies are available to prevent repeated reactions among patients who are reaction-prone. These include leukocyte reduction for the prevention of FNHTR, cell washing for the prevention of allergic and anaphylactic reactions and possibly some FNHTRs, and various premedication regimens.

**Table 53-3.** Overlapping Signs and Symptoms of Transfusion Reactions

| Sign or Symptom     | Possible Reaction                                          | Most Likely > Less Likely Blood Component*     |
|---------------------|------------------------------------------------------------|------------------------------------------------|
| Fever, chills       | Febrile nonhemolytic<br>Septic<br>Acute hemolytic<br>TRALI | Platelets (especially septic)<br>>RBCs >Plasma |
| Urticaria, pruritus | Allergic<br>Anaphylactic                                   | Plasma >Platelets >RBCs                        |
| Dyspnea             | TACO<br>TRALI<br>Anaphylactic                              | Any                                            |
| Hypotension         | Septic<br>Hypotensive<br>Acute hemolytic<br>Anaphylactic   | Platelets >plasma >RBCs                        |

\*Granulocytes would very likely cause febrile reactions and dyspnea. However, granulocytes are not listed in the table because they are infrequently transfused compared to other blood components.

RBCs = Red Blood Cells; TRALI = transfusion-related acute lung injury; TACO = transfusion-associated circulatory overload.

## Disclaimer

The author has disclosed no conflicts of interest.

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Transfusion-associated graft-vs-host disease (TA-GVHD) is a phenomenon that can occur after blood transfusion in which donor T cells, responding to proteins on host cells, proliferate and target host organs, primarily the skin, liver, intestinal tract, and marrow. Although this complication is uncommon, considerable effort has been devoted to GVHD prevention, because of the lack of effective treatment and very high mortality rate. Immunocompromised patients are at greatest risk for TA-GVHD; therefore, prevention strategies initially focused on this population. More recently it has become recognized that TA-GVHD can develop in the apparently immunocompetent transfusion recipient. Patients most likely to develop TA-GVHD include very low birthweight premature infants, critically ill patients receiving extracorporeal membrane oxygenation, patients who require cardiopulmonary bypass, and recipients of blood transfusions from family members. This chapter outlines the pathophysiology and clinical manifestations of TA-GVHD, defines the risk factors, and identifies measures to prevent TA-GVHD.

### Pathophysiology of GVHD

In 1966, Billingham postulated that three requirements existed for the development of GVHD: the graft must contain immunologically competent cells, there must be antigenic differences between the donor and the recipient, and the recipient must be unable to mount an effective immunologic response to eradicate the transplanted immunologically competent cells.<sup>1</sup> T cells are the immunologically competent cells that induce GVHD and can be transferred from one person to another in a variety of clinical settings, including blood component transfusions and organ and marrow transplants. Under most circumstances, the recipient immune system is able to eliminate the transferred T cells. However, when the recipient is immunosuppressed (either from

inherited or acquired immunodeficiency or induced immunosuppression, as is the case in transplant recipients) GVHD can develop. Immunocompetent individuals can develop TA-GVHD, although this is a very rare event.

Most of our understanding of the pathophysiology of GVHD comes from the hematopoietic stem cell transplant (HSCT) setting. In HSCT, presentation of host antigen to donor cells often occurs in the context of host tissue damage and inflammation, from a combination of factors including the underlying disease, infection, and the HSCT conditioning regimen, which often includes high-dose chemotherapy and radiation therapy. Donor cells are transplanted into an environment of ubiquitous foreign antigens, activated cytokines, enhanced expression of adhesion molecules, and cell surface recognition molecules. Donor cells that participate in the resulting immune process include T cells, natural killer (NK) cells, and monocytes.

A modern paradigm for GVHD development considers three consecutive phases: 1) the presentation of host protein to donor T cells by antigen-presenting cells (APCs); 2) donor T-cell activation, proliferation, and migration; and 3) host target tissue damage (Fig 54-1). Host APCs are both necessary and sufficient to stimulate donor T cells to proliferate in response to host antigen.<sup>2-5</sup> T-cell receptors (TCRs) recognize fragments of proteins known as minor histocompatibility antigens bound to Class I and II major histocompatibility complex (MHC) molecules on the surface of APCs. The Class I MHC molecules (HLA-A, -B, -C) stimulate CD8+ T cells, while the Class II MHC molecules (HLA-DR, -DP, -DQ) present to CD4+ T cells.<sup>6,7</sup> The additional presence of “danger signals”—such as lipopolysaccharide (LPS) or inflammatory cytokines—help APCs activate T cells<sup>8,9</sup> and the presence/absence of “danger signals” may make the difference between an immune response and tolerance.<sup>10</sup>

Activated T cells can have proinflammatory (Th1) or anti-inflammatory (Th2) phenotypes. Th1 cells secrete interleukin (IL)-2, interferon (IFN)- $\gamma$ , and tumor necrosis factor alpha (TNF- $\alpha$ ) while Th2 cells secrete IL-4, IL-5, IL-10, and IL-13.<sup>11</sup> Th1 cells are important in the pathogenesis of acute GVHD and targeting these cells reduced acute GVHD in an experimental

## GVHD pathology

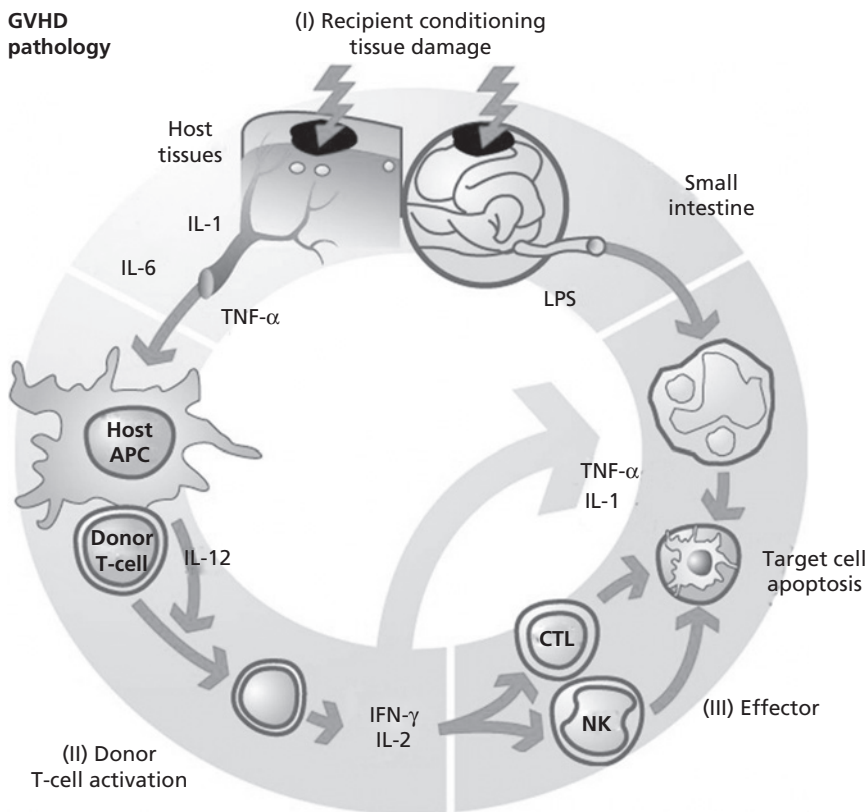


**Figure 54-1.** The pathophysiology of acute GVHD.

In Step I, the conditioning regimen of chemotherapy, radiotherapy, or both leads to host tissue damage, especially in the intestines. This allows translocation of lipopolysaccharide (LPS) from the intestine into the circulation, stimulating the secretion of inflammatory cytokines, including interleukin (IL)-1, IL-6, and tumor necrosis factor alpha (TNF- $\alpha$ ). In Step II, cytokine-stimulated host antigen-presenting cells (APCs) present host antigen to donor T cells, resulting in activation of donor T cells with a predominance of the Th1 phenotype that secrete interferon- $\gamma$  (IFN- $\gamma$ ) and IL-2. In Step III, effector cells, cytotoxic T cells, and natural killer cells, induce target cell apoptosis both by direct cell-mediated lysis and the secretion of inflammatory cytokines that independently induce target cell apoptosis.

model.<sup>12</sup> In clinical practice, decreasing IL-2 production by donor T cells is a primary target of current GVHD prophylaxis strategies by using the calcineurin inhibitors cyclosporine or tacrolimus. In contrast, the Th2 cytokine IL-10 plays a key role in suppression of immune responses and may have a role in regulating acute GVHD severity.<sup>13,14</sup> Regulatory T cells, a third subset of T cells, also influence the development of GVHD. In animal models, adding regulatory T cells to the donor graft suppresses the proliferation of conventional T cells, and prevents GVHD.<sup>15</sup> Murine regulatory T cells, which are identifiable by Foxp3 expression, normally constitute 5% of the CD4+ T-cell population. These cells secrete IL-10 and transforming growth factor beta (TGF- $\beta$ ), which inhibit the immune response. The regulatory T cells also inhibit APCs by a contact-dependent pathway.<sup>15</sup>

Migration of T cells from lymphoid tissues to target organs is controlled by chemokines. In experimental GVHD, proinflammatory chemokines, such as macrophage inflammatory protein-1 alpha (MIP-1 $\alpha$ ), CCL2-5, CXCL2, CXCL9-11, CCL27 are overexpressed. The chemokines may have organ-specific roles. For example, CXCR3+ cells cause acute GVHD in the liver and intestine.<sup>16</sup> In animal models, migration of CCR5+ regulatory T cells to lymphoid tissues and target organs is necessary for GVHD control.<sup>17</sup>

Experimental and clinical data indicate that target organ injury results from both soluble inflammatory mediators and cell-mediated cytotoxicity. The principal effector mechanism by which cytotoxic T lymphocytes (CTLs) and NK cells lyse target cells are the Fas/Fas

ligand (FasL) and perforin/granzyme pathways.<sup>18,19</sup> Fas is an apoptosis-signaling receptor molecule on the surface of a number of different cells. When FasL binds to Fas, a death-inducing signaling complex is formed, which results in the subsequent activation of caspases.<sup>20</sup> CTLs and NK cells store perforin with granzyme in cytotoxic granules. Perforin inserts itself into the target cell membrane, forming pores that allow granzymes to enter the cells and induce apoptosis through various downstream effector pathways.<sup>21</sup> Inflammatory cytokines synergize with CTLs, resulting in amplification of local tissue injury and development of target organ dysfunction. A key inflammatory cytokine, especially for intestinal GVHD, is TNF- $\alpha$ , which plays a major role in the amplification and propagation of the “cytokine storm” characteristic of acute GVHD developing early after HSCT.<sup>22</sup> TNF- $\alpha$ , which is produced by both donor and host cells, promotes GVHD and target organ damage by three different ways: first, it activates dendritic cells and enhances alloantigen presentation; second, it recruits effector cells to target organs via the induction of inflammatory chemokines; and third, it directly causes tissue necrosis, as its name suggests.<sup>23-25</sup>

## Features of TA-GVHD

### Incidence of TA-GVHD

The incidence of TA-GVHD is unknown because the diagnosis can be missed; other conditions, such as infection or drug

reactions, may have similar clinical features. However, changes in practice appear to have lowered the already low incidence even further. The use of cellular components that have been gamma irradiated to 25 Gy appears to offer essentially complete protection to patients known to be at high risk for TA-GVHD.<sup>26</sup> In the 10 year period from 1996 to 2005, 13 cases of TA-GVHD were reported via a national identification program. The implementation of universal leukocyte reduction in the United Kingdom (UK) in 1999 appears to have resulted in a statistically significant further reduction of TA-GVHD; of the 13 cases, 11 occurred in patients who received non-leukocyte-reduced (LR) components and only two occurred following transfusion of LR components. No cases of TA-GVHD have been reported in the UK since 2001.<sup>27</sup>

### Risk Factors for Developing TA-GVHD

As noted above, the transfusion of viable T cells is necessary to develop TA-GVHD. GVHD can develop after transplantation of as few as  $4 \times 10^3$  T cells/kg recipient weight,<sup>28</sup> although other studies indicate that acute GVHD is not seen when fewer than  $1$  to  $5 \times 10^5$  T cells/kg recipient weight are transplanted.<sup>29,30</sup> The average unit of Red Blood Cells (RBCs) contains approximately  $2 \times 10^9$  leukocytes, each whole-blood-derived platelet unit contains approximately  $4 \times 10^7$  leukocytes, and apheresis platelets contain approximately  $1 \times 10^8$  leukocytes.<sup>31</sup> Leukocyte reduction reduces the number of passenger T cells by 2 to 3 logs, but has not been able to completely prevent TA-GVHD.<sup>32-34</sup> A 2-log reduction of viable leukocytes can be achieved by washing red cells<sup>34</sup> and a 2 to 3 log reduction is attained by freezing-deglycerolization.<sup>34,35</sup> For obvious reasons, leukocyte reduction is not performed with therapeutic white cell transfusions, and these can lead to TA-GVHD.<sup>36,37</sup> Irradiating these cells can prevent GVHD (as discussed below), but donor leukocyte transfusions given to induce a graft-vs-leukemia effect following post-HSCT relapse are not irradiated and GVHD often results.<sup>38</sup> TA-GVHD has not been reported with the use of cell-free plasma products, including Fresh Frozen Plasma, because the freezing-thawing process destroys T cells.<sup>39</sup> Unfrozen plasma has caused TA-GVHD,<sup>40</sup> but is now rarely used.

Because TA-GVHD is caused by the engraftment of viable T lymphocytes, the primary risk factor for development of TA-GVHD is an inability to reject donor T lymphocytes. The mechanisms are the same as those discussed for HSCT GVHD. It is of interest that TA-GVHD has been reported in only one patient with human immunodeficiency virus (HIV)/AIDS.<sup>41</sup> Despite their immunodeficiency, HIV/AIDS patients appear to be resistant to engraftment of donor T lymphocytes, for unclear reasons.<sup>42</sup>

### Fetuses and Neonates

Maternal lymphocytes regularly cross over into fetal circulation, but under most circumstances GVHD does not develop, indicating that the fetus has immunologic defense mechanisms against maternal lymphocyte engraftment.<sup>43</sup> Nonstimulated bidirectional mixed lymphocyte cultures have shown that neonatal lymphocytes specifically dominate against maternal lymphocytes, which may partly account for the neonatal protection against

maternal lymphocytes.<sup>44</sup> However, the fetal immune system does not completely protect against adult lymphocytes as demonstrated by the development of TA-GVHD following intrauterine transfusion (IUT). Liley performed the first successful intraperitoneal IUT to treat hemolytic disease in 1963.<sup>45</sup> Not long afterwards, it became apparent that IUT could lead to TA-GVHD.<sup>46,47</sup> As a result, transfusion with LR and irradiated blood was initiated to prevent TA-GVHD. However, up to one-third of recipients of nonirradiated, non-LR transfusions engrafted donor leukocytes, without any GVHD or other apparent disorder of the immune system, and these donor leukocytes were detectable up to 25 years following IUT.<sup>48</sup> The mechanisms behind this selective tolerance for the donor cells are unclear, but may involve downregulation of molecules essential for immune recognition. It is also possible that tolerance between the fetus and the donor may develop because of the following:<sup>49</sup>

1. Lack of host immune surveillance because of immaturity.
2. Inadequate expression of target antigens in fetal tissue.
3. Presence of maternal lymphocytes that protect against GVHD.

TA-GVHD in neonates transfused postnatally is very rare. Most cases of TA-GVHD in immunocompetent newborns occur following transfusions from haploidentical or homozygous donors. Therefore, it is not surprising that most reports come from Japan, where there is a high degree of HLA homozygosity. Another risk factor may be prematurity. In one Japanese study, 20 of 27 newborns who developed TA-GVHD were premature.<sup>50</sup> Most of the newborns received a blood component from a relative. TA-GVHD developed a median of 28 days from transfusion with fever typically the presenting sign, followed by GVHD rash. The mortality rate was 100%. The implementation of routine irradiation of blood and blood components before transfusion in newborns; congenital immunodeficient recipients; elderly recipients; and cardiac, cancer, and severe trauma surgery patients, has reduced the number of TA-GVHD cases in Japan to just a few cases in recent years.<sup>51</sup>

### TA-GVHD in Immunodeficient Patients

TA-GVHD occurs in T-cell deficient patients, who lack the ability to reject the donor T cells, usually caused by a congenital immunodeficiency syndrome, treatment for malignancy, or solid-organ transplantation (Table 54-1).

### Congenital Immunodeficiency Syndromes

TA-GVHD has been reported in a wide array of congenital immunodeficiency syndromes, including severe combined immunodeficiency (SCID) syndrome, Wiskott-Aldrich syndrome, purine nucleoside phosphorylase deficiency, and DiGeorge syndrome.<sup>52-56</sup> As expected, a common feature to these disease entities is abnormal or absent T-cell function.

### Malignancies

TA-GVHD has been reported to occur following nonirradiated transfusion in patients undergoing treatment for cancer.<sup>37,57-69</sup> It is not clear if the presence of malignancy alone increases the

**Table 54-1.** Patient Groups at Risk for TA-GVHD**Neonates**

- Intrauterine transfusion (IUT)
- Postnatal transfusion in recipients of IUT
- Very low-birthweight premature neonates
- Neonatal alloimmune thrombocytopenia

**Congenital Immunodeficiency**

- Severe combined immunodeficiency
- Wiskott-Aldrich syndrome
- DiGeorge syndrome
- Other T-cell defects

**Malignancies and Immunosuppression Caused by Treatment**

- Leukemia and lymphoma
- Neuroblastoma
- Rhabdomyosarcoma
- Other solid tumors

**Purine Analog Therapy**

- Fludarabine

**Hematopoietic Cell Transplantation**

- Allogeneic
- Autologous
- Syngeneic

**Solid-Organ Transplant**

- Lung
- Liver
- Heart
- Kidney
- Pancreas

**Surgery**

- Cardiac surgery
- Extracorporeal membrane oxygenation

risk of TA-GVHD, or if the problem is caused by the immunosuppressive effects of treatment. Most cases of TA-GVHD have been reported for patients with hematologic malignancies such as leukemia or lymphoma but there have been rare cases of TA-GVHD in patients with solid tumors such as neuroblastoma and rhabdomyosarcoma. This observation suggests that it is the immunosuppressive effect of the cancer treatment that increases the risk of TA-GVHD because antileukemia and antilymphoma treatment tends to be more immunosuppressive than treatment for solid tumors. However, there has been no systematic attempt to correlate the extent or duration of therapy to the development of TA-GVHD. One specific chemotherapeutic agent, the purine analog fludarabine, has been associated with TA-GVHD.<sup>69-71</sup> Fludarabine very efficiently depletes T cells and is used to treat a variety of indolent lymphomas, especially chronic lymphocytic leukemia.<sup>72</sup> Immunosuppressive therapy with fludarabine, even in the absence of malignancy, has also been associated with the development of TA-GVHD.<sup>73</sup> Because fludarabine is often used as a single agent, the connection between its use and TA-GVHD may be easier to discern. It appears prudent to consider the use of any immunosuppressive chemotherapy agent to be a risk factor for TA-GVHD. In any event, the routine use of preventive

measures against TA-GVHD in cancer patients regardless of diagnosis or treatment appears to have reduced the incidence of TA-GVHD. A review of the literature finds very few reports of TA-GVHD in cancer patients since 1997,<sup>70,74,75</sup> although this may, in part, reflect underreporting.

Autologous stem cell collections are also at risk for contamination with donor lymphocytes if nonirradiated blood components are given during the marrow or peripheral stem cell collection. This can lead to the subsequent development of TA-GVHD following reinfusion of the autologous stem cells and passenger donor lymphocytes.<sup>76</sup> A syndrome consisting of features reminiscent of GVHD, occurring generally around the time of engraftment following autologous stem cell transplant, has been reported.<sup>77</sup> This syndrome, termed engraftment syndrome, presents with noninfectious fever, skin rash, capillary leak, pulmonary infiltrates, and hypoxia. Biopsies of the skin rash are often equivocal with respect to establishing a diagnosis. TA-GVHD has not been ruled out in all of these cases, but it is generally felt that this syndrome represents changes associated with proinflammatory cytokine release around the time of engraftment. Likewise, the phenomena of autologous GVHD, which occurs after autologous stem cell transplant, can be induced by administration of cyclosporin A for a short period following transplantation, leading to the generation of autoreactive T cells.<sup>78</sup> One way to distinguish between autologous GVHD and TA-GVHD is to demonstrate engraftment of transfused donor lymphocytes through techniques such as HLA Class I or II antigen differences or other genetic variations such as differences in short tandem repeat.<sup>79</sup>

**Solid-Organ Transplantation**

GVHD in solid-organ transplantation is usually caused by donor lymphocytes transported with the donor organ but can also occur from transfusions given when the recipient is intensely immunosuppressed to prevent organ rejection. GVHD has been reported following cadaveric liver, heart, kidney, and pancreas-spleen transplants.<sup>80-86</sup> In one study,<sup>82</sup> 12 cases of GVHD occurred in 1082 liver transplants performed at a single institution. GVHD symptoms (fever, skin rash, diarrhea, or pancytopenia) typically developed within 2 to 6 weeks after the transplant. Mortality was over 90%.

Another risk factor for GVHD following organ transplant is HLA homology between the donor and the recipient.<sup>87,88</sup> Matching at the HLA-B locus appears to particularly dispose patients to the development of GVHD following liver transplantation.<sup>89</sup>

**TA-GVHD in Immunocompetent Patients****HLA Homozygosity**

One risk factor for TA-GVHD in immunocompetent patients is transfusion from a donor who is HLA homologous to the recipient. This situation is most likely to occur when the donor is a family member,<sup>90-92</sup> but there are also many reports from Japan, where a genetically homogenous population increases the risk of transfusion from a partially HLA-matched random

donor.<sup>91,93</sup> The transfusion recipient is less likely to reject donor lymphocytes when the donor is homozygous for an HLA haplotype for which the recipient is heterozygous. In this situation, the donor does not carry HLA antigens that trigger allogeneic recognition on the part of the recipient and thus are able to engraft. However, the recipient possesses HLA antigens that are foreign to the donor, and thus alloreaction culminating in TA-GVHD can occur.<sup>94,95</sup> Most recent reports of TA-GVHD come from settings or locales where blood irradiation is not routine.<sup>95</sup>

### Surgery

TA-GVHD has been reported in immunocompetent individuals undergoing cardiac surgery.<sup>96-98</sup> Cardiopulmonary bypass may increase the risk of TA-GVHD through the induction of transient immunodeficiency as evidenced by reduced mitogenic lymphocyte transformation and reduced production of the pro-immune response cytokines, TNF- $\alpha$ , and IL-2.<sup>99-101</sup> Likewise, TA-GVHD has been seen following extracorporeal membrane oxygenation.<sup>102</sup> An additional risk factor contributing to the development of TA-GVHD is transfusion of cellular blood components from donors who share HLA antigens with the recipient, such as related donors and donor-recipient dyads from areas with low HLA diversity, such as Japan.<sup>97,99,103,104</sup>

### Fresh Blood

Another risk factor appears to be the use of freshly donated blood. In a series of 2686 consecutive adult patients who underwent cardiac surgery between 1980 and 1996, four cases of TA-GVHD developed, all following transfusion of cellular blood components donated within 48 hours.<sup>104</sup> Transfusion of fresh blood components has been associated with TA-GVHD in other studies, as well.<sup>94,95,105</sup> Fresh blood presumably predisposes patients to TA-GVHD because it contains maximal numbers of viable lymphocytes. During storage the number of viable lymphocytes declines<sup>106</sup> as does the expression of cell-surface lymphocyte activation antigens.<sup>107</sup> The decrease in cell surface lymphocyte antigen expression directly correlates with a reduced ability to activate T cells as measured by a mixed lymphocyte culture by Day 3 of storage. This finding helps explain the rarity of TA-GVHD following transfusion of blood that is more than 4 days old.<sup>103,107</sup>

In summary, in immunocompetent recipients, the factors most likely to lead to TA-GVHD are as follows:

1. HLA-homozygous donor to haploidentical recipient, particularly likely if the donor is a relative or transfusion takes place in a region with low HLA genetic diversity.
2. Cardiac bypass.
3. Use of blood less than 4 days old.

## Clinical Presentation and Diagnosis of TA-GVHD

Unlike GVHD occurring after HSCT, which can take weeks to months to develop (median onset: 23 days),<sup>108-111</sup> GVHD following transfusion typically occurs sooner, usually within a

**Table 54-2.** Comparison of GVHD Following Hematopoietic Stem Cell Transplantation with Transfusion-Associated GVHD\*

| Manifestation                | HSCT GVHD             | TA-GVHD             |
|------------------------------|-----------------------|---------------------|
| Median onset (range)         | 23 days (12-100 days) | 10 days (2-30 days) |
| Fever                        | Often                 | Usually             |
| Skin rash                    | +                     | +                   |
| Liver involvement            | +/-                   | +                   |
| Gastrointestinal involvement | +/-                   | +/-                 |
| Pancytopenia                 | Rare                  | Almost always       |
| Occurrence                   | 25%-50%               | Rare                |
| Response to therapy          | 35%-50%               | Rare                |
| Mortality                    | 10%-25%               | 90%-100%            |

\*Modified from Brubaker.<sup>113</sup>

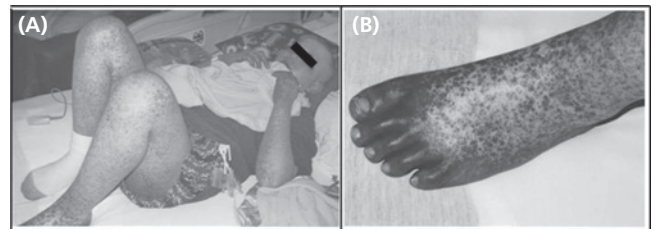


**Figure 54-2.** A patient with Stage III skin GVHD following hematopoietic cell transplantation. (A) Generalized skin erythema with the characteristic maculopapular rash. (B) Two days later, the rash is more intense with areas of confluence.

few days, but up to 4 weeks (median onset: 10 days) from transfusion<sup>95,112</sup> (Table 54-2<sup>113</sup>). Fever is often the first symptom, but is soon followed by target organ involvement of skin, gastrointestinal tract, and/or the liver.<sup>114</sup> The host hematopoietic system is also a target in GVHD resulting in pancytopenia, a major contributor to mortality, in the transfusion-associated version.<sup>115</sup> Pancytopenia is less commonly seen in GVHD following allogeneic HSCT because the transplanted donor hematopoietic stem cells are spared from donor attack. Consistent with the observation that the hematopoietic system is a specific target in TA-GVHD, newborns with TA-GVHD reveal striking thymic damage.<sup>50</sup> There is no grading system for pancytopenia, but other target organs are staged using a scale developed for HSCT-associated GVHD (see Chapter 35).

The skin is usually the first organ involved. The characteristic maculopapular rash is pruritic, often involves the palms of the hands and soles of the feet, and spreads throughout the body (Fig 54-2). In severe cases the skin may blister and ulcerate.<sup>111,114</sup> Apoptosis at the base of epidermal rete pegs is a characteristic pathologic finding. Other features include dyskeratosis, exocytosis of lymphocytes, satellite lymphocytes adjacent to dyskeratotic epidermal keratinocytes, and a perivascular lymphocytic infiltration in the dermis.<sup>116,117</sup>



Gastrointestinal tract GVHD usually presents as diarrhea but may also include vomiting, anorexia, and/or abdominal pain when severe.<sup>116</sup> The diarrhea of GVHD is secretory and often voluminous (greater than 2 L/day). Bleeding occurs as a result of mucosal ulceration and can lead to hemorrhagic death.<sup>118</sup> Endoscopic examination with biopsies can confirm gastrointestinal tract involvement. Histologic features include lymphocytic infiltration, patchy ulcerations, apoptosis of crypt epithelial cells, crypt abscesses, and loss as well as flattening of the surface epithelium.<sup>119,120</sup>

Liver involvement in GVHD is characterized by laboratory and clinical features of cholestasis, with elevated bilirubin levels in the blood and clinical jaundice.<sup>111</sup> The histologic features of hepatic GVHD are bile duct damage, portal and lobular inflammation, endothelialitis, and canalicular cholestasis.<sup>121</sup> However, the liver is rarely biopsied because thrombocytopenia greatly increases the risks of the procedure.

Diagnosis of TA-GVHD is usually made on clinical grounds, but detection of donor lymphocytes in the recipient's circulating blood confirms the diagnosis. Techniques to establish donor lymphocyte engraftment are based on genetic differences between the donor and the host and include chromosome differences by standard cytogenetics,<sup>122</sup> fluorescent *in situ* hybridization of a biopsy,<sup>123</sup> detection of small genetic differences in highly polymorphic regions,<sup>79</sup> and differences in tissue typing.<sup>61,67,97</sup> A source of recipient chromosomes or DNA is necessary and can be obtained by buccal scraping or skin biopsy.

## Treatment of GVHD

Transfusion-associated GVHD carries a very poor prognosis with mortality rates over 90% despite aggressive treatment that has included agents such as high-dose steroids, azathioprine, methotrexate, cyclosporine, intravenous immunoglobulin, and growth factors.<sup>39,74,105</sup> The principles of treatment for TA-GVHD come primarily from the HSCT literature. Steroids, with their potent antilymphocyte and antiinflammatory activity, are the gold standard for treatment of GVHD. However, steroid therapy results in durable complete or partial remission in only around 50% of patients, primarily those with less severe GVHD.<sup>108,124</sup> Antithymocyte globulin (ATG) has been used together with steroids to improve outcome. In a prospective randomized study of 100 patients, the combination of steroids and ATG was no more effective than steroids alone as front-line therapy for GVHD; however, there was a better-than-expected response rate to steroids alone.<sup>124</sup> In a retrospective study, 1-year survival was better when ATG was added within 4 to 7 days of steroid therapy for those patients who showed early signs of steroid resistance.<sup>126</sup> Other studies have not shown benefit when ATG is added to steroid therapy to treat GVHD.<sup>127,128</sup> Because ATG increases the risk of infection in patients with GVHD, it is not used routinely.<sup>125,127,128</sup> This increased risk of infection is of particular importance in the treatment of TA-GVHD, because death from overwhelming infection is common.<sup>94-96</sup>

One treatment that may be less immunosuppressive is extracorporeal photopheresis (ECP). During ECP, the patient's white cells are collected by apheresis, incubated with the DNA-intercalating agent, 8-methoxypsoralen, exposed to ultraviolet A (UVA) light, and returned to the patient. ECP is known to induce cellular apoptosis, which has strong antiinflammatory effects in a number of systems, including prevention of rejection of solid-organ grafts.<sup>129</sup> A Phase II study of ECP in steroid-dependent or steroid-refractory GVHD showed complete and durable resolution of GVHD in a large majority of patients. High response rates in the skin (82%), liver (61%), and intestine (61%) translated into 50% long-term survival in this very high-risk group.<sup>130</sup> Another possible strategy to treat GVHD is inhibition of the inflammatory cytokine TNF- $\alpha$ , a molecule that plays a critical role in the development of GVHD as discussed earlier. A Phase II trial of etanercept, a solubilized dimeric TNF-receptor-2, showed significant efficacy when added to systemic steroids as primary therapy for acute GVHD. Seventy percent of patients had complete resolution of all GVHD symptoms within 1 month, with 80% complete responses in the gastrointestinal tract and the skin. The authors also showed that plasma levels of TNF-receptor-1 were a significant biomarker for clinical GVHD.<sup>131</sup>

Given the very high mortality from TA-GVHD, only a few cases of successful treatment have been reported. An infant with X-linked SCID developed TA-GVHD following non-irradiated blood transfusion. The TA-GVHD was initially controlled with high-dose steroid therapy. A haploidentical transplant from his mother was initially rejected by the engrafted blood donor T lymphocytes, but on second transplant engraftment was achieved with eventual eradication of blood transfusion donor T cells. The blood donor, patient, and marrow donor all shared an HLA haplotype.<sup>54</sup> In two cases, GVHD was successfully treated by infusion of patient lymphocytes.<sup>71,132</sup> Anti-CD3 treatment, together with cyclosporine, showed effectiveness in one case.<sup>133</sup> A spontaneous remission has also been reported.<sup>134</sup>

## Prevention of TA-GVHD

### $\gamma$ -Irradiation

Although TA-GVHD is a preventable disease, there remains considerable controversy regarding the optimal prevention strategies, primarily because of the rarity of the diagnosis. Treating cellular blood components with 25 Gy  $\gamma$ -irradiation reliably inactivates donor lymphocytes through cross-linkage of DNA, and prevents TA-GVHD.<sup>39,135</sup> Irradiation does not damage platelets or granulocytes, and therefore can be performed safely at any time following the collection.<sup>112</sup> However, irradiation does shorten the shelf life of red cell components and it is recommended that irradiated red cells be used within 28 days from the time of irradiation.<sup>39</sup> The effects of irradiation on red cells include increased plasma membrane permeability, with potassium leakage, hemolysis, and problems with adenosine triphosphate maintenance.<sup>136</sup> Prestorage leukocyte reduction may lessen some of the effects of



irradiation on red cell storage parameters.<sup>137</sup> Irradiation incurs additional cost and is not available at all hospitals. Therefore, although universal irradiation of all cellular components would eliminate TA-GVHD, this strategy has not been adopted in all countries.<sup>138,139</sup>

In Japan, TA-GVHD has been a particularly significant issue because of the genetically homogenous population. In 2000, Japanese experts recommended blood component irradiation for the following identified risk situations: transfusions from blood relatives, granulocyte transfusions, and all transfusions to immunodeficient recipients including any cancer patients, newborns, elderly patients, and those undergoing cardiac surgery.<sup>51</sup> Subsequently, universal blood component irradiation was adopted in Japan.<sup>139</sup> In the United States and Europe, there is no well-established consensus on the indications for blood irradiation. Most transfusion services perform their own assessment of the risk and develop a list of indications for which blood irradiation is provided routinely. The indications most frequently included in such a list are: transfusions from blood relatives; intrauterine transfusions; granulocyte transfusions (except when given to patients with congenital neutrophil disorders); patients with congenital cellular immunodeficiency syndromes, Hodgkin disease, and other leukemias and lymphomas; patients taking fludarabine; and patients undergoing HSCT. Many authorities irradiate fresh blood (less than 24 hours old) when used for transfusion. Other indications are assessed differently at different institutions.

Blood irradiation can be performed with commercial irradiators designed specifically for this purpose. The most appropriate dose for blood component irradiation is 25 Gy at midplane.<sup>135,140</sup> Commercial irradiators incorporate one to four gamma ray sources, generally cesium-137, and deliver 300 to 1000 cGy per minute.<sup>141</sup> Linear accelerators and cobalt-60 can also be used as a source of  $\gamma$ -irradiation.<sup>135,141</sup> Alternatives to cesium-137 as a radiation source are preferred because of concerns about security risks, despite the increased costs of such alternatives. Radioactive cesium chloride used in blood irradiators and other medical equipment is widely used in significant quantities, is soluble and easily dispersible, and often lacks secure disposal options.<sup>142</sup>

### Nonirradiation Prevention Strategies

Leukocyte reduction has been shown to reduce the risk of TA-GVHD, especially in a genetically diverse population,<sup>27</sup> but is not a substitute for irradiation in at-risk populations. Photochemical treatment using psoralens and long-wavelength ultraviolet irradiation inactivates T cells and has also been shown to inactivate blood-borne pathogens, such as cytomegalovirus.<sup>143,144</sup>

### Summary

Transfusion-associated GVHD is a rare and preventable disease that occurs when sufficient numbers of viable T cells are transfused into a susceptible host. Immunodeficient states, either inherited or

acquired, are well recognized as a risk factor, but TA-GVHD can develop in immunocompetent patients, especially if fresh blood components, with their high numbers of viable leukocytes, are transfused. Immunocompetent recipients are generally only at risk if they receive HLA haploidentical T cells, which is most likely to happen if the donor is a relative, or if the patient lives in a region with low HLA genetic diversity. TA-GVHD usually develops within days of the transfusion and follows a fulminant course with death from severe pancytopenia and infection. The disease is generally recognized by classical features of rash, diarrhea, and liver dysfunction. Irradiation of blood components is highly effective at preventing this disease, but is not universally employed. Careful attention must be paid to identifying at-risk patients, because, once established, the disease is almost always fatal.

### Disclaimer

The authors have disclosed no conflicts of interest.

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Tissue iron overload inevitably results in patients who receive regular red cell transfusions for congenital or acquired anemias. Iron is deposited initially in the liver and monocyte-macrophage (reticuloendothelial) system, but as these tissues become saturated, iron begins to deposit in other organs such as the endocrine glands and the heart. The body lacks an effective means of eliminating this excess iron, and without therapy, cirrhosis, heart disease, diabetes, and other disorders develop; death is usually the result of cardiac failure. Transfusional iron overload, with its resulting morbidity and mortality, is an important health issue in patients of all ages. Children with thalassemia major, or other inherited disorders such as Diamond-Blackfan anemia (DBA), congenital dyserythropoietic anemia, and congenital aplastic anemia are dependent on regular red cell transfusions to maintain their well being. Children with sickle cell disease who are found to be at increased risk of stroke may be placed on a chronic transfusion program to reduce this risk substantially.<sup>1</sup> Some adults with aplastic anemia or myelodysplastic syndrome (MDS) may also become dependent on regular red cell transfusions. In addition, with chemotherapeutic regimens becoming more intense and myelosuppressive, patients undergoing treatment for various forms of cancer, particularly leukemias and lymphomas, may also receive a large number of red cell transfusions. Some of these individuals may also undergo stem cell transplantation, during the course of which they may receive numerous such transfusions. With advances in the medical management of some of these conditions, including improved chelation regimens, patients also have a better survival probability, thus increasing the number of individuals in whom transfusional iron overload requires close medical attention. Until recently, the most long-term experience in the pathogenesis and management of transfusional iron overload came from studies in patients with thalassemia major. Although these individuals differ from other

transfusion-dependent patients in some important ways, experience with patients with thalassemia helps guide many of the recommendations made here.<sup>2</sup>

### Pathophysiology

Iron is an essential nutrient required by every cell. It is present in the human body in hemoglobin, myoglobin, and several mitochondrial respiratory enzymes. It serves as a carrier for oxygen and electrons and acts as a catalyst for a variety of oxygenation reactions. It is able to perform these functions in part because of its ability to reversibly and readily cycle between its ferrous and ferric forms. This very property also makes it potentially toxic and capable of producing free radicals, which can cause cellular damage (Fig 55-1).

The normal body iron concentration is approximately 40 to 50 mg/kg body weight; women have lower amounts and men somewhat higher.<sup>4</sup> Three-quarters of this iron, about 30 mg/kg, is contained in the circulating red cell compartment as hemoglobin. Approximately 5 to 6 mg/kg is present in functional form in a variety of heme compounds (myoglobin and cytochromes) and iron-dependent enzymes. The remainder (5 mg/kg in women, 10-12 mg/kg in men) is held in reserve in two primary

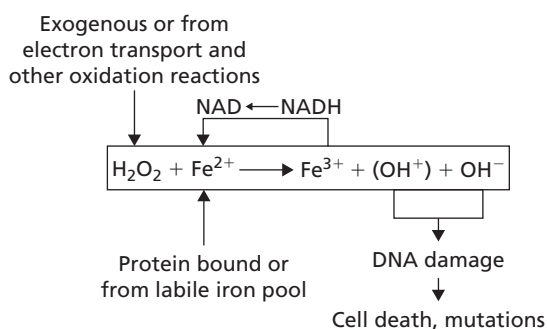


**Figure 55-1.** Iron-induced oxidative tissue damage via the Fenton Reaction.<sup>3</sup>

storage forms (ferritin and hemosiderin) in the liver, marrow, spleen, and muscle—readily available when required for erythropoiesis. Iron balance is maintained by controlling iron absorption; iron stores and iron absorption are reciprocally related so that as stores increase, absorption declines. These processes are closely managed by the iron regulatory “hormone” hepcidin,<sup>5</sup> which plays a critical role in iron metabolism. It regulates the absorption of iron from the gastrointestinal tract through its effect on levels of ferroportin, the protein responsible for transporting iron out of the enterocyte. Red cells are normally broken down by cells of the monocyte-macrophage system, and the iron contained in hemoglobin is usually returned via transferrin to the erythroid precursors in the marrow, for recycling into new red cells. Hepcidin controls the release of iron from macrophages, an effect also mediated by ferroportin. In the healthy host, when the body has adequate or increased amounts of iron, hepcidin is upregulated, iron absorption from the intestine is inhibited, and iron is sequestered in its storage sites, the macrophages and hepatocytes. The body lacks any effective mechanism for the excretion of excess iron. Iron exchange is limited so that the adult male absorbs and loses only about 0.01 mg/kg/day.

Iron overload results from repeated blood transfusion, excessive iron absorption from the gastrointestinal tract, or of a combination of these processes.<sup>4</sup> In patients with severe congenital anemias (such as the thalassemias, DBA, or congenital dyserythropoietic anemia), death from severe anemia in infancy is averted by a regular transfusion program which, if adequate, allows for normal growth and development during the first decade of life. Individuals with MDS or aplastic anemia may begin transfusions later in life. As in healthy individuals, macrophages process senescent red cells (transfused in these patients) and extract iron from heme. With no effective means for the excretion of this iron, there is gradual tissue accumulation of this iron, which eventually exceeds the body's capacity for safe storage. As the amount of iron increases, there is virtually no change in the amount that is contained within the functional or transport (transferrin-bound) pools, and almost all of the excess iron is stored. Initially, the iron is sequestered in the cells of the monocyte-macrophage system, but with continued accumulation, iron spills over and is deposited in other tissues, usually the liver (hepatocytes), heart, pancreas, and other endocrine organs.

Transfusion-dependent individuals may be broadly categorized as those having ineffective erythropoiesis (such as individuals with thalassemia or some forms of sideroblastic anemia), those with effective erythropoiesis (such as individuals with sickle cell anemia or hereditary spherocytosis), and those with decreased or absent erythropoiesis (such as patients with aplastic anemia or DBA). The “effectiveness” of erythropoiesis, with the ability to incorporate iron into hemoglobin in mature red cells, is a critical determinant of the pathophysiology of iron loading in transfusion-dependent patients. Once transfused red cells are phagocytosed by macrophages, hepcidin levels determine the fate of iron released from heme. If hepcidin levels are high, more iron remains sequestered in these storage cells, and less is released for

potential deposit in other tissues. Iron stored in the hepatocyte is similarly retained. In contrast, if hepcidin levels are low, more iron is released from these cells and may be available for deposition in other parenchymal tissues. Thus, hepcidin helps determine the partition and internal redistribution of the excess iron between the monocyte-macrophage and parenchymal sites,<sup>6</sup> another crucial factor in tissue toxicity.

Ineffective erythropoiesis—and, to a lesser degree, anemia—result in a downregulation of hepcidin expression and enhanced absorption of iron from the intestine.<sup>7</sup> Patients with thalassemia major or intermedia, paradoxically have relatively low levels of hepcidin even in the presence of iron overload, as a result of their marked ineffective erythropoiesis. In patients with sickle cell disease, who have intact erythropoiesis, hepcidin expression varies inversely with erythropoietic drive,<sup>8</sup> with untransfused patients excreting less hepcidin in the urine. Similarly, patients with DBA or aplastic anemia, who have almost no erythropoiesis, likely have appropriately elevated hepcidin levels when iron loading occurs. Thus, patients with thalassemia major or intermedia not only have higher iron burdens because of increased intestinal absorption, but also have a greater risk of parenchymal tissue deposition than the latter groups. In fact, patients with thalassemia intermedia who have received very few or no red cell transfusions may have profound iron overload because of marked hepcidin inhibition<sup>7</sup> as a result of their ineffective erythropoiesis. In patients with thalassemia major or intermedia, regular transfusion suppresses ineffective erythropoiesis to some extent, and hepcidin levels may rise somewhat. However, the levels remain inappropriately low in relation to the higher body iron burden, resulting in iron loading through gastrointestinal absorption. Recently, a growth factor (GDF15) has been identified that is secreted during erythroblast maturation, and suppresses hepcidin production. There is increased expression of this factor in thalassemia, likely because of the ineffective erythropoiesis.<sup>9</sup> In contrast, levels of this factor are not elevated in patients with homozygous sickle cell anemia. Therefore, although the volume of red cells transfused is similar, variability in the hepcidin response to the underlying erythropoietic status produces differences in the partition and distribution of the excess iron.

While hepcidin levels are critical in modifying the pathophysiology of iron loading in transfusion-dependent patients, several other variables are important in determining the morbidity related to the increased body iron burden. These include 1) the age at which transfusion therapy began, 2) the duration of transfusion therapy, and 3) the initiation and maintenance of effective chelation. Additional modifying factors include other genetic determinants, alcohol use, coexisting viral hepatitis, and other drugs and medications that the patient may be taking. The complex interplay between the underlying disorder and these factors in each individual plays a key role in the pathophysiology of iron toxicity.

Even though the body has the ability to safely store increased amounts of iron, deposition in nonstorage tissues eventually occurs as the body iron burden rises. Without treatment,

patients with thalassemia, who begin transfusions in infancy, develop growth retardation, diabetes, and other endocrine disturbances in the first decade of life, and death from iron loading of the myocardium occurs during the second decade. Patients with sickle cell disease, who receive regular transfusions to prevent the occurrence of strokes, may begin these transfusions later in childhood, and may achieve normal growth and sexual maturation before the threshold for tissue damage is reached. A comparative study of regularly transfused patients with thalassemia major and sickle cell disease with similar iron burdens showed that the latter group had fewer cardiac and endocrine complications.<sup>10-12</sup> The patients with sickle cell disease began regular transfusions at a later age, and had been transfused for approximately half as long as the thalassemia patients, possibly contributing to the observed differences in clinical manifestations. These observations emphasize the relevance of the age at which transfusions are begun, particularly with regard to growth, development, and sexual maturation. As the duration of transfusion increases, severe iron loading may occur in all transfusion-dependent patients who are not effectively chelated. Diabetes and cardiac disease may develop, the latter remaining the major cause for morbidity in all of these patients.

As the body accumulates iron from transfused red cells, chelation therapy is usually begun to prevent deposition and damage to parenchymal tissues. Just as the rate of uptake of iron by these tissues is influenced by transfusion parameters and hepcidin levels, the variability of the chelation regimen also plays an important role. The timing of initiation, choice of chelator and dosing, and compliance with the regimen are some of these variables. When transfusion and chelation are occurring simultaneously, there is likely a complex and dynamic balance between transfusional iron loading and chelator-induced iron purging.<sup>13</sup> Different organs may take up iron at different rates and likely have different thresholds for when tissue damage occurs. It has been demonstrated that in transfused and chelated patients with thalassemia and sickle cell disease, the relationship the liver iron concentration (LIC) and myocardial iron levels is nonlinear and variable.<sup>13,14</sup>

In summary, the pathophysiology of iron metabolism in the condition underlying the transfusion-dependent anemia, as well as the factors directly related to the initiation, intensity, duration, and effectiveness of both the transfusion regimen and the chelation regimen, contribute to the pathology of transfusional iron overload.

Storage iron is present in the body predominantly in two high-molecular-weight forms, ferritin and hemosiderin. Both forms are stained on pathologic specimens by Prussian blue, via the Pearl's reaction. Hemosiderin is insoluble and is deposited in aggregates of varying sizes, while ferritin is soluble and more homogeneously distributed within cells. Under physiologic conditions, iron is distributed approximately equally between these storage forms, but as the body's iron load increases, the proportion stored as hemosiderin increases dramatically. A small amount of iron is present in a low-molecular-weight intracellular

pool—likely the most toxic form of iron, because of its propensity for producing free radicals, which cause oxidative damage. It is likely that the ferritin fraction is in closer equilibrium with the low-molecular-weight iron pool.<sup>15</sup> As the magnitude of iron excess grows, some stored iron may be mobilized into the circulation from macrophages or hepatocytes. As transferrin is increasingly saturated, some plasma non-transferrin-bound iron (NTBI) appears. This iron may be more easily taken up by parenchymal tissues that do not normally store iron, leading to toxicity. Thus, it is critical to ensure that effective chelation therapy is ongoing, to bind this iron and prevent its entry into cells, thereby avoiding organ damage.

Histopathologic examination of the liver in mild transfusional iron overload reveals predominant Kupffer cell iron deposition, with preserved hepatocyte architecture and no fibrosis. As iron loading continues, the hepatocytes also demonstrate increased iron uptake; there is some disruption of the architecture and some scarring/fibrosis. Until this point, these changes are reversible with appropriate chelation. With massive overload, there is bridging fibrosis, and progression to cirrhosis, which may or may not be reversible.<sup>16</sup> Myocardial iron distribution is heterogeneous, with more in the left heart than the right, more in the ventricles than the atria, and more in the epicardium than the endocardium.<sup>17</sup> It has been suggested, based on studies in the iron-loaded gerbil model, that iron in myocardial fibers may cause impaired repolarization of the myocytes.<sup>18</sup> This may interfere with generation and orderly propagation of the electrical impulse and lead to a variety of arrhythmias. With progressive loading, fibers may not contract normally, and a restrictive cardiomyopathy with impaired filling and diastolic dysfunction may result. This can eventually lead to congestive heart failure. Heart failure is reversible in part, and with improved chelation, cardiac function may be recovered substantially.<sup>19,20</sup> Therefore, long-term monitoring of transfused and chelated patients should include serial measurement of myocardial iron deposition as well as the LIC.

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## Transfusional Iron Burden

The total amount of iron that enters the body via red cell transfusion can be estimated. Because the magnitude of the iron burden is critical in the development of complications, it is of great importance to maintain accurate records, for each patient, of the amount of red cells transfused. The total amount of transfused red cells is calculated as the total amount of blood in mL multiplied by the hematocrit of each unit (as a percentage) divided by 100. Each mL of red cells contains 1.08 mg of iron, and the total amount of iron introduced (Kin) is calculated as follows:

$$\text{Kin} = (\text{total amount of red cells transfused, in mL}) \times 1.08$$

Alternatively, if the exact volume is not available, the iron burden can be estimated from the number of units transfused.



Each Red Blood Cell (RBC) unit transfused contains 200 to 250 mg of iron. The total iron burden can be calculated with use of one of these methods. Transfusion-dependent patients usually require 200 to 300 mL/kg/year of blood, an amount equivalent to 0.25 to 0.40 mg/kg/day of iron.

With no physiologic means of excreting this excess iron, and the inapplicability of phlebotomy in patients who are regularly transfused, these individuals must receive iron chelation therapy in order to facilitate excretion. A minimum chelator-induced excretion of 0.25 to 0.40 mg/kg/day would maintain iron balance in such individuals. Higher levels of excretion would be necessary produce a negative iron balance.

Iron balance should be monitored during chelation therapy to assess the efficacy of such treatment. The total body iron (Us) at any point in time (t) may be extrapolated from the LIC (in mg/g dry weight) using the formula published by Angelucci et al<sup>21</sup> as follows:

$$Us(t) = 10.6 \times LIC \times (\text{body weight in kg})$$

For patients who are being chelated as well, there is an ongoing change in the body iron balance as the iron entering the body through transfused red cells is removed by the chelator. In these situations, it is important to monitor the total body iron excretion between LIC measurements. This can be calculated based on the amount of red cells transfused ( $K_{in}$  = iron introduced in mg), and on the changes in total body iron between measurements of liver iron concentrations at baseline ( $t_0$ ) and a later time (t), expressed as milligrams of iron excreted per day.<sup>22</sup>

$$\text{Total body iron excretion} = (K_{in} + [Us(t_0) - Us(t)]) / (t - t_0)$$

## Clinical Features

Although iron deposition in tissues begins soon after the initiation of regular transfusions, signs and symptoms of iron toxicity usually occur after a few years of iron loading. By then the usual storage sites for iron have been almost saturated, and there is deposition in other tissues that do not normally store iron. Organs suffer damage, often irreversible, resulting in significant morbidity. Early death is sometimes seen, generally as a result of cardiac failure.<sup>23,24</sup> In a recent cross-sectional study of patients with thalassemia born after 1970 in Italy, it was found that 7% had heart failure, 6% had diabetes, 11% had hypothyroidism, and 55% had hypogonadism.<sup>25</sup> A third of these patients had died, the cause of death in 68% being heart failure.

Liver disease of transfusional iron overload may be manifested as hepatomegaly, abnormalities in function, fibrosis (which may lead to scarring, bridging fibrosis, and micronodular regeneration and cirrhosis), and hepatocellular carcinoma.<sup>25,26</sup> Patients are asymptomatic, or have mild to moderate icterus, and with progression, symptoms and signs of cirrhosis and hepatic failure.

Heart failure remains the leading cause of mortality in transfusional iron overload. Unfortunately, symptoms do not appear until the myocardium has large amounts of deposited iron, and routine testing by conventional echocardiography and Holter monitoring early in the course almost always yields normal results. With progressive deposition, patients manifest symptoms. Early signs may include a variety of arrhythmias including supraventricular or ventricular tachycardias, premature or extra systoles, heart block, and atrial fibrillation. Contractile dysfunction affects diastole early,<sup>28</sup> likely the reason that conventional echocardiography, which assesses mainly systolic function, indicates “normal” function. The onset of cardiac failure is late, and often sudden, and is an ominous sign. Both complications are often refractory to treatment. Therefore, prevention of myocardial iron loading is critical.

In patients who begin receiving transfusions in early childhood, the developing endocrine organs are particularly susceptible to iron deposition if effective chelation is not initiated at the appropriate time. The anterior pituitary is most often involved, resulting in slow growth, delayed sexual maturation, and often infertility, the latter being the result of direct iron deposition in the gonads as well. Supplemental growth hormone or sex hormone therapy is often required to treat these complications. The pancreatic islet cells are also susceptible to the toxic effects of iron. Impaired insulin secretion and abnormal glucose tolerance usually precedes the development of insulin-dependent (type 1) diabetes and may occur in highly loaded individuals at any age.<sup>29,30</sup> Older males may experience dysfunction in sexual performance, and females may develop secondary amenorrhea related to gonadal iron deposition and disturbances in sex hormone secretion. Thyroid dysfunction, although uncommon, may also manifest at any age.<sup>26</sup>

Other manifestations include arthropathy and skin pigmentation, which are directly related to iron deposition in these tissues. Patients with marked hemosiderosis appear bronzed initially, and with progression, may appear darker, or grayish. Complicating the manifestations of iron toxicity are the clinical signs and symptoms of chelator side effects, which are described below.

## Measurement of Iron Burden

The goal of therapy for individuals with transfusional iron overload is to maintain iron balance at low levels of tissue iron, thereby preventing the development of overload and complications. When there is little or no ineffective erythropoiesis, simply keeping track of the volume of red cells transfused provides a measure of the body iron burden in transfused patients who are not being chelated. Different tissues load iron at different rates and the balance between loading via transfusion and removal by chelation is also a determinant in the differential deposition of iron. Thus, monitoring iron deposition in any one organ is not representative of the others. The ideal means of monitoring iron overload is to periodically assess tissue iron levels in each of the

different organs affected, but this is currently feasible only in the liver and heart.

The LIC has been used to assess the body iron burden. In patients with thalassemia major, who had been cured by stem cell transplantation, phlebotomy was used as a means of removing excess iron that had accumulated from years of red cell transfusions. A serial decline in the LIC correlated well ( $R = 0.98$ ;  $p < 0.001$ ) with the amount of iron removed by phlebotomy, supporting the idea that the LIC is a reflection of total body iron load.<sup>21</sup> Patients with thalassemia who had LICs of 6 to 13 mg/g dry weight had an almost twofold risk of progression of fibrosis; in those with liver iron concentrations of 13 to 41 mg/g dry weight, the hazard rate was almost ninefold.<sup>31</sup> Coexisting infection with the hepatitis C virus (HCV) independently increased the risk for progression of fibrosis threefold. These observations confirmed the results of previous studies: LIC  $> 7$  mg/g dry weight is associated with an increased risk of hepatic fibrosis, diabetes, and other complications of iron overload.<sup>32</sup> Maintaining the LIC in the suggested “ideal range” of 3 to 7 mg/g dry weight in regularly transfused and chelated patients should minimize iron deposition in nonstorage parenchymal sites, and prevent significant toxicity. Because correlation between hepatic and cardiac iron concentrations is not satisfactory,<sup>13,14</sup> independent assessment of myocardial iron should be performed, as discussed in the next section.

Whereas the LIC can be measured directly by biopsy or noninvasive means, direct quantification of myocardial iron is not feasible. A surrogate for cardiac iron burden, myocardial T2\*, has been shown to correlate with function,<sup>33</sup> and is currently used in several centers. In the absence of methods for assessing iron deposition in other tissues such as the pancreas and anterior pituitary, current ideal monitoring includes periodic assessment of hepatic and myocardial iron deposition.

Serial measurement of the LIC over time is an accurate and reliable means of monitoring the progression of iron loading and the efficacy of chelation therapy. The ideal method for measuring the LIC is one that is safe, accurate, noninvasive, and valid over a wide range of concentrations. Magnetic resonance (MR)-based methods meet almost all of these criteria, although access to centers offering them may be limited.

The reference method has been the quantitative chemical estimation of nonheme iron in liver tissue obtained by biopsy. To obtain accurate results by this method, several requirements must be met: the patient should not have cirrhosis or focal lesions, the biopsy must weigh at least 1 mg dry weight (or 4 mg wet weight), and should be processed using strictly iron-free methods. Serial biopsies would ideally allow monitoring not only of the rate of iron loading and the efficacy of chelation therapy in the liver, but also the development and rate of progression of fibrosis or scarring. Liver biopsy is an invasive procedure with considerable risks, and is not ideal for the frequent measurement of LIC that is necessary for the monitoring of regularly transfused and chelated individuals.

A recent workshop by the National Institute of Diabetes and Digestive and Kidney Diseases concluded that a clinical need

was evident for quantitative means of measuring body storage iron that are noninvasive, safe, accurate, and readily available to improve the diagnosis and management of patients with iron overload, including hereditary hemochromatosis (HH), thalassemia major, sickle cell disease, aplastic anemia, myelodysplasia, and other disorders.<sup>34</sup> Several noninvasive methods have been used to estimate the LIC. These include direct methods such as magnetic biosusceptometry, and indirect methods such as MR.

Recently, there has been tremendous interest in using MR techniques to study tissue iron.<sup>35-37</sup> A brief description of the application of these techniques is presented here; a more in-depth analysis has recently been published.<sup>38</sup> Hemosiderin and ferritin iron affect the relaxation of hydrogen atoms present in the nuclei of tissue water molecules. Initial limitations in the technology have been overcome and more sensitive instruments capable of measuring more rapid signal decay (very short relaxation times) are now used to quantify hepatic and cardiac iron concentration. Several such methods have been used to assess the hepatic iron concentration.<sup>39-41</sup> Two widely used techniques are a gradient-echo T2\*-based sequence that measures the signal intensity ratio of liver to skeletal muscle,<sup>42</sup> and a stimulated spin-echo R2 sequence-based scan (<http://www.resonancehealth.com>).<sup>43</sup> Measurements of hepatic iron concentration obtained by this technique correlate well with hepatic iron chemically estimated directly from liver tissue. T2\* sequences have been used to quantify hepatic iron, but the correlation is less robust.<sup>33</sup> The relationship between estimates of LIC by MR methods and the serum ferritin has been variable.

Iron concentrations in the myocardium are much lower than those in the liver, and the iron is distributed nonhomogeneously in different areas of the heart. A multi-echo spin-echo T2\* technique is the most widely used method of assessing myocardial iron.<sup>44</sup> Although no direct correlation of T2\* with tissue iron has been demonstrated in humans, data are available from the iron-loaded gerbil model.<sup>45</sup> All individuals with clinical cardiac disease had T2\* measures of  $< 20$  msec, and all subjects with T2\* values above 20 msec had normal cardiac function.<sup>33</sup> The threshold for “severe” cardiac disease has also been reduced to a T2\* value of 8 msec, below which there is very high risk for development of cardiac disease.<sup>46</sup> Several studies have compared the efficacy of different chelators in removing myocardial iron using this method.<sup>20,47,48</sup>

Magnetic resonance techniques are being developed to study iron deposition and its effects in other organs as well, including the anterior pituitary and pancreas, enabling better surveillance for development of complications and closer monitoring of the efficacy of chelation in preventing or ameliorating iron deposition.

Magnetic biosusceptometry has been validated to be a safe, accurate, and noninvasive method of quantitatively measuring the LIC over a wide range of body iron burdens. The principle is that storage iron (both hemosiderin and ferritin) is paramagnetic, ie, when a steady magnetic field is applied, these molecules will induce a field change that is proportional to the number of

iron atoms present. An extremely sensitive sensor, or superconducting quantum interference device (SQUID), measures this response. A strong linear correlation ( $R = 0.99$ ) between LIC determined by biopsy and by SQUID susceptometry has validated this technique.<sup>49</sup> SQUID susceptometers have been used extensively in studies of iron-loaded individuals to assess iron burden and guide chelation therapy.<sup>22,32,50-52</sup> Use of this method has been limited because of the complexity and high cost of building, installing, and maintaining the instrument. Currently, only four centers worldwide are using this technique. Other limitations include use in individuals who are obese, are very small, or have metal implants or devices that may not be removed before the study. In addition, susceptometric methods cannot be used to quantify iron in other organs that may be loaded with iron, such as the heart, pancreas, and other endocrine organs. A new high-transition temperature device, cooled using liquid nitrogen and with better resolution of the signal from surrounding tissues, is under development.<sup>53</sup>

Traditionally, the serum ferritin level has been used as an indirect estimate of the body iron burden. Several studies have shown a significant correlation between the serum ferritin level and the LIC estimated by biopsy in patients with thalassemia.<sup>32,54-55</sup> However, the coefficient of correlation in most of these was poor, with marked scatter around the correlation line. A recent study in patients with sickle cell disease also showed poor correlation between serum ferritin levels and the LIC measured in biopsy tissue in patients with sickle cell disease.<sup>57</sup> There are several reasons for this variability in the serum ferritin level, which does not necessarily reflect a change in the body iron burden. Ferritin is well-known to be an acute-phase reactant. Serum levels may be increased by infection, inflammation, hepatic dysfunction, tissue injury, hemolysis, and decreased when there is chronic hypoxia or a deficiency of ascorbic acid. Thus, it is at best an indirect marker of iron overload. Although practical and easy to measure, ferritin is not reliable as an indicator of the total body iron burden or as a parameter for the monitoring of chelator efficacy, and does not in any way identify the differential iron loading that may exist between the liver and the heart.

Without the wide availability of noninvasive measures of hepatic and cardiac iron, there is still reliance on the serum ferritin level as a means of monitoring the iron burden and the efficacy of chelation. However, it may actually be misleading in individual patients, remaining stable in the face of rising body iron burden and ongoing deposition in the heart and endocrine organs. Similarly, measurements of serum iron, transferrin or transferrin saturation, and urinary iron excretion do not provide reliable indication of the level of body iron stores. Plasma A NTBI has been suggested to be a marker of the “toxic iron pool,”<sup>58,59</sup> but its measurement is very complex and is currently still used for research purposes only. These limitations in serum measurement of iron-related moieties underscore the need for assessment of iron in specific tissues.

As discussed previously, there is variability in iron deposition between the heart and the liver, as a result of the changing

balance between transfusional iron loading and chelation.<sup>13,14</sup> Thus, optimal monitoring of iron burden should include assessment of both—the hepatic iron concentration by biopsy, MR, or susceptometry and the myocardial iron by the surrogate T2\* estimate. These noninvasive monitoring methods are not yet widely available, but special effort should be devoted to securing such testing for patients in order to chelate them effectively and prevent the development of complications.

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## Management

The primary goal of therapy is to prevent tissue deposition of excess iron, thereby preventing organ damage and the resulting morbidity and mortality. Maintaining safe levels of tissue iron requires achieving a balance between the amount of iron entering the body and that being removed. Unlike management of HH patients, phlebotomy to remove excess iron is not an option for those with transfusional iron overload unless the patient has successfully undergone stem cell transplantation, or has completed treatment with intensive chemotherapy regimens. If transfusion therapy is ongoing, iron-overloaded patients must be treated with a chelating agent capable of sequestering iron and permitting its excretion from the body. As discussed previously, a minimum chelator-induced excretion of 0.25 to 0.40 mg/kg/day is necessary to maintain iron balance. The ideal chelator should form a high-affinity 1:1 uncharged complex with iron; be able to chelate intracellular iron; be orally effective, with a long half-life, high chelator efficiency, and low toxicity profile; and be effective in removing iron from (or preventing deposition in) all organs that may be affected with long-term usage. Such a chelator is not yet available. Chelators in current use are described in brief below; more detailed reviews of chelation are available elsewhere.<sup>60,61</sup>

### Chelating Agents

Deferoxamine mesylate (Desferal, Novartis, East Hanover, NJ) (Fig 55-2), a naturally occurring trihydroxamic acid produced by *Streptomyces pilosus*, was first introduced almost 40 years ago and has been found to be a generally safe and effective means of managing iron overload, ameliorating or preventing iron-induced organ damage, and reducing morbidity and mortality. In fact, patients with thalassemia major who were effectively chelated survived significantly longer than those whose chelation was ineffective.<sup>62</sup> Deferoxamine is a hexadentate chelator that forms a charged 1:1 complex with iron. This complex does not readily enter or leave cells and is excreted almost equally by the liver in bile and by the kidneys. Unfortunately, deferoxamine is not absorbed intact when taken orally, has a very short half-life (approximately 15 minutes), and must be administered by subcutaneous infusion using a portable syringe pump over 8 to 12 hours daily for maximal chelation efficiency.<sup>63</sup> It has been shown that levels of NTBI (the form of iron that more easily enters parenchymal cells) rise in the plasma within 16 hours

of the end of the infusion,<sup>63</sup> and maintaining chelator levels in the body is critical to prevent toxic effects of this iron. Patients who receive the drug in this manner often have pain, experience swelling and redness at the site of infusion, and may develop injection abscesses. Rare toxicities occur when deferoxamine is present in excess of chelatable iron, including effects on hearing (usually high-frequency hearing loss) and vision (leading to blindness in severe instances).<sup>63,64</sup> For all of these reasons, compliance with this regimen is not very good, and morbidity from organ deposition and dysfunction is often the result. The dosage range for deferoxamine is between 30 and 50 mg/kg administered over 8 to 12 hours by subcutaneous infusion five to seven times a week.<sup>64</sup> In severely loaded individuals, such as those with iron induced cardiac disease,<sup>19</sup> it may be administered by continuous intravenous infusion, although dosing above 50 mg/kg/day does not increase iron excretion further,<sup>63</sup> and may result in toxicity. Although cardiac function typically improves relatively quickly (2-3 months), such continuous therapy often has to be maintained for many months or even years before there is a substantial decline in the LIC or improvement in the myocardial T2\*.<sup>20</sup>

Deferiprone (1,2-dimethyl-3-hydroxypyridin-4-one) (Fig 55-2) is a bidentate chelator that forms an uncharged 3:1 complex with iron, enabling it to cross membranes easily and remove toxic iron from cells. It is effective orally, but has a short half-life (<2 hours) and must be taken three or four times a day. Excretion occurs in the urine. It is less effective than deferoxamine in reducing the LIC, but seems to have greater effectiveness in removing myocardial iron.<sup>47</sup> A retrospective study of patients who had undergone long-term treatment with deferiprone showed that there were significantly fewer cardiac events in those patients compared to patients who had been treated with deferoxamine alone.<sup>24</sup> Serious idiosyncratic complications have been reported with its use, including neutropenia (up to 5%), agranulocytosis (up to 0.5%), the development of an erosive

arthropathy (5% to >20%), and the development of a neurologic syndrome of cerebellar and psychomotor retardation.<sup>65</sup> In spite of adherence to the suggested weekly monitoring of blood counts, one death from agranulocytosis has been reported.<sup>66</sup> A Canadian study described progression of hepatic fibrosis in several patients who were undergoing long-term therapy.<sup>67</sup> A subsequent study did not confirm this finding<sup>68</sup> but has been criticized.<sup>69</sup> Deferiprone is prescribed at 75 to 100 mg/kg/day divided into three doses, with careful monitoring of blood counts every week. The ability of this drug to move across the cell membrane led to the idea of using it in combination with deferoxamine, with deferiprone acting as a siphon to bind iron and transport it extracellularly. Although one study showed that patients treated with combination deferoxamine and deferiprone showed significantly greater improvements in T2\* than those on deferoxamine alone,<sup>48</sup> the significance of this finding is limited by the small number of subjects and the short duration of evaluation. This drug is not approved for use in the United States, and the conclusion of a recent independent review by the Cochrane Collaboration<sup>70</sup> was that “deferiprone is indicated for the treatment of iron overload in patients with thalassemia major when deferoxamine is contraindicated or inadequate.”

Deferasirox (Exjade, Novartis) (Fig 55-2) is a tridentate N-substituted bis-hydroxyphenyltriazole chelator that forms a 2:1 complex with iron that is primarily excreted in bile. With a plasma half-life of 12 to 16 hours, once-daily administration means the drug is present in the circulation throughout the day, enabling constant effective scavenging of iron. Phase III studies of this agent, comparing it to deferoxamine, have only recently been reported.<sup>32</sup> At the suggested and subsequently approved starting dose of 20 mg/kg, most patients were not able to achieve negative iron balance in this study, although most but not all did appear to maintain their iron balance. Although it was able to cause negative iron balance (reduction in LIC) in the majority of patients at doses of 30 mg/kg/day, there was a high incidence (up to 39%) of hepatic and/or renal toxicity at this dose level. Other side effects include gastrointestinal upset (15%), rash (11%), and, rarely, neutropenia. Patients who are taking this agent should be monitored carefully for hepatic and renal function, and appropriate dose adjustments should be made. As yet, there are no published data on the efficacy of deferasirox in removing myocardial iron, but studies are in progress. Trials using 30 to 40 mg/kg/day of deferasirox are under way for highly iron-loaded patients who are intolerant to deferoxamine. Combination trials with deferoxamine and deferasirox are currently being designed.

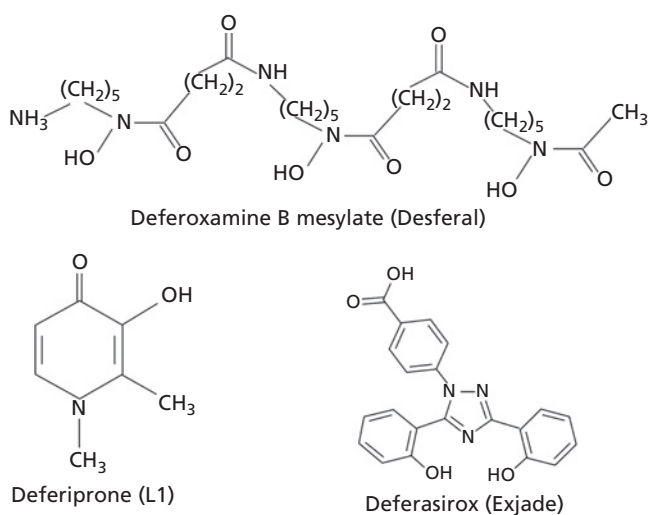


**Figure 55-2.** Chemical structure of the different chelator molecules.

### Initiation of Chelation

The optimal range recommended for maintaining the LIC is 3 to 7 mg/g dry weight, as discussed previously. When this range is reached after regular transfusion, chelation therapy should be initiated. There are no immediate clinically apparent consequences of not instituting chelation when the patient reaches this range of iron overload, because such complications usually result at higher levels and after more prolonged loading.

The goal of therapy is to prevent these complications and chelation should be instituted well before they occur. The range for initiation of chelation is usually reached when the total transfused red cell volume reaches 150 to 180 mL/kg. If records of transfused volumes are not available, the LIC (as measured by biopsy) or noninvasive methods (such as SQUID or MR) should be used to guide the decision. In children in whom iron is required for growth, aggressive chelation could potentially reduce the amount of available iron. A conservative LIC of 4 to 5 mg/g dry weight is usually used as the threshold for initiating chelation (Table 55-1). Although an oral chelating agent would be much preferred by patients compared to a parenterally administered one, there is still not enough experience or data with regard to the long-term use of deferasirox as a first-line agent, and deferiprone is recommended only if deferoxamine is contraindicated.<sup>71</sup> Deferoxamine is still recommended as the choice for initiation of chelation.<sup>71</sup> Patient education, compliance, and preference should also be taken into consideration.

### Compliance

Compliance with chelation therapy is the primary determinant of morbidity and survival in such patients, and the responsibility of the medical team caring for such patients in reinforcing this cannot be emphasized enough. If compliance with chelation is poor, iron loading exceeds removal and iron accumulates first in the liver and then in the other parenchymal tissues including the heart. A recent meta-analysis of 18 studies involving patients with thalassemia confirmed that noncompliance results in significant cardiac and endocrine morbidity, and increased risk of death from heart disease.<sup>72</sup> When transfusion and chelation are begun in early childhood, it is usually the responsibility of the parent to ensure that the chelation regimen is followed. In adolescence and young adulthood, when this responsibility shifts to the patient, various distractions and life-changing events may result in poor compliance. Often a lack of physical symptoms in spite of significant iron overload lulls such individuals into a false sense of wellness and invulnerability. This is a particularly critical time for all caregivers to monitor these patients very carefully. Similarly, older individuals who have begun regular transfusion

later in life may feel that it is unnecessary to chelate because the onset of symptoms may be years later. Education and constant reinforcement is necessary to ensure compliance and reduce morbidity and mortality.

Patients undergoing chelation therapy should be vigilant for symptoms and signs of infection, particularly if they have undergone splenectomy. Chelation may be temporarily suspended if there is any suspicion of a bacterial or fungal illness, until this is appropriately managed.

### Intensification of Chelation

Noncompliance resulting in a positive iron balance and an increase in the LIC or shortening of the T2\*, would necessitate changes in the chelation regimen to prevent progression of organ damage. The compliance record should be closely examined and consultation with an expert in the management of iron overload should be undertaken before intensification of chelation is begun. Dosage of deferoxamine can be increased to a maximum of 50 mg/kg/day, beyond which there seems to be no additional increment in urinary iron excretion.<sup>63</sup> Higher doses have also been associated with the development of a pulmonary syndrome.<sup>73</sup> Increasing the frequency of subcutaneous administration to 7 days per week is a more effective strategy (Table 55-1). For patients with symptomatic heart disease, continuous intravenous chelation with deferoxamine at 50 mg/kg/day has been shown to be effective in reversing heart failure, improving cardiac function, and reducing the LIC more effectively<sup>19</sup> (Table 55-1). Generally, an indwelling catheter is required, because deferoxamine may cause sclerosis of peripheral veins. Close monitoring for toxicity and efficacy is necessary. Because noncompliance is the primary cause of severe loading, this regimen may not be acceptable to the patient. Although a combination of deferoxamine and deferiprone has been reported to be effective in reducing the iron burden in the liver and the heart,<sup>48</sup> the number of patients in the study was small, and the duration of follow-up was short. Longer prospective studies with larger numbers of patients are required before combination therapy can be recommended. Results of long-term, prospective, randomized trials of higher doses of deferasirox are not available. Intravenous deferoxamine

**Table 55-1.** Guidelines for Chelation Therapy

|                                                                      | Indication                                                                        | Choice of Product/Regimen                                                                                                                                                          |
|----------------------------------------------------------------------|-----------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Initiation of chelation                                              | LIC 3-7 mg Fe/g dry weight, or total red cell volume transfused 150-180 mL/kg     | Deferoxamine 35-40 mg/kg by subcutaneous infusion over 8-12 hours five to seven times/week; only if deferoxamine is unacceptable, deferasirox 20 mg/kg/day on empty stomach, daily |
| Intensification (in conjunction with expert consultation and advice) | LIC 8-15 mg Fe/g dry weight, or T2* <20 msec, without clinical cardiac disease    | Deferoxamine 50 mg/kg/day by subcutaneous infusion daily                                                                                                                           |
|                                                                      | LIC >15 mg Fe/g dry weight, or T2* <10 msec, or onset of clinical cardiac disease | Deferoxamine 50 mg/kg/day by continuous infusion via central venous catheter                                                                                                       |

LIC = liver iron concentration.

as described above should also be considered for any patient with a severe iron burden, corresponding to LICs above 15 mg/g dry weight even in the absence of clinical cardiac deposition, and for those with myocardial T2\* values below 10 msec.

### Monitoring Chelation Therapy

Once chelation is begun, regular noninvasive assessment of iron deposition in the heart (MRI T2\*) and liver (MRI R2 or SQUID) every 12 to 18 months is recommended (Table 55-2) to ensure the efficacy of chelation and give warning of rising levels, which may be associated with significant iron toxicity. Higher LICs predispose to progression of fibrosis,<sup>31</sup> and the development of cirrhosis; keeping the iron level in the ideal range of 3 to 7 mg/g dry weight is an important preventive measure. Simultaneously, monitoring the cardiac T2\* for myocardial deposition is also critical. A liver biopsy is indicated if there is coexisting hepatitis or liver dysfunction suggestive of fibrosis or progression to cirrhosis. Adjustments in the chelation regimen should be based on changes in the LIC or the cardiac T2\*. More frequent measurements may be indicated after such changes have been made to ensure optimal efficacy of the regimen.

In addition to monitoring iron burden and adherence to chelation therapy, organ function must also be monitored for the development of abnormalities related to the transfusion and

chelation regimen (Table 55-2). The ideal method for assessing cardiac function is MR assessment of chamber dimensions, ventricular filling, and ejection fractions. This would provide indicators of systolic as well as diastolic function. Conventional echocardiography does not provide adequate assessment of diastolic function, and is suboptimal. Cardiac dysfunction in iron-induced cardiac disease is mainly diastolic and systolic function is preserved until late in the course of the iron-induced restrictive cardiomyopathy. Normal results often lead to a false sense of well being and noncompliance with chelation. Serial measurements may provide useful information on trends and may signal the need for closer evaluation by MRI.<sup>74</sup> Despite preserved global function, tissue Doppler echocardiography-detected regional wall motion abnormalities represent an early sign of cardiac disease.<sup>75</sup> These special echocardiographic techniques may be of limited use, but annual MR-based cardiac function assessment, performed at the same time as T2\* measurement, is the ideal method of monitoring. Holter monitoring for development of arrhythmias, is also recommended annually. Regular assessment of hepatic and renal function should also be part of comprehensive care. In addition to screening for transfusion-associated viral infections, frequent assessment is also indicated to monitor for chelator toxicity. Comprehensive annual endocrine evaluation including thyroid and parathyroid

**Table 55-2.** Recommended Comprehensive Evaluation of Regularly Transfused and Chelated Individuals

| System                  | Test                                                                                                                                        | Frequency                                                                                                                                      |
|-------------------------|---------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------|
| Monitoring of iron load | <ul style="list-style-type: none"> <li>LIC by biopsy, SQUID, or MRI</li> </ul>                                                              | Every 12-18 months; more frequently if iron burden is high and patient is undergoing intensive chelation                                       |
| Cardiovascular          | <ul style="list-style-type: none"> <li>Cardiac T2*</li> </ul>                                                                               | Annually                                                                                                                                       |
|                         | <ul style="list-style-type: none"> <li>MRI for assessment of function (echocardiography with tissue Doppler if MRI not possible)</li> </ul> | Annually                                                                                                                                       |
| Hepatobiliary           | <ul style="list-style-type: none"> <li>Holter monitoring</li> </ul>                                                                         | Annually                                                                                                                                       |
|                         | <ul style="list-style-type: none"> <li>Bilirubin levels and AST/ALT monitoring</li> </ul>                                                   | Every transfusion visit if patient is taking deferasirox or if HBV or HCV infection is active; less frequent if patient is taking deferoxamine |
| Renal                   | <ul style="list-style-type: none"> <li>HBV, HCV serology</li> </ul>                                                                         | Annually                                                                                                                                       |
|                         | <ul style="list-style-type: none"> <li>BUN and serum creatinine</li> </ul>                                                                  | Every transfusion visit if patient is taking deferoxamine or deferasirox                                                                       |
| Endocrine               | <ul style="list-style-type: none"> <li>Thyroid and parathyroid function</li> </ul>                                                          | Annually                                                                                                                                       |
|                         | <ul style="list-style-type: none"> <li>Bone density</li> </ul>                                                                              | Annually                                                                                                                                       |
|                         | <ul style="list-style-type: none"> <li>Glucose tolerance</li> </ul>                                                                         | Annually                                                                                                                                       |
|                         | <ul style="list-style-type: none"> <li>hGH, testicular and ovarian function</li> </ul>                                                      | Based on age and clinical indications                                                                                                          |
| Other                   | <ul style="list-style-type: none"> <li>HIV serology</li> </ul>                                                                              | Annually                                                                                                                                       |
|                         | <ul style="list-style-type: none"> <li>Vision and hearing</li> </ul>                                                                        | Annually if patient is taking deferoxamine                                                                                                     |
|                         | <ul style="list-style-type: none"> <li>Pulmonary function</li> </ul>                                                                        | Every 2 years                                                                                                                                  |

LIC = liver iron concentration; SQUID = superconducting quantum interference device; MRI = magnetic resonance imaging; AST = aspartate transaminase; ALT = alanine transaminase; HBV = hepatitis B virus; HCV = hepatitis C virus; BUN = blood urea nitrogen; hGH = human growth hormone; HIV = human immunodeficiency virus.

function, bone density assessment, glucose tolerance, and gonadal function testing are recommended, especially for growing children and adolescents. Vision and hearing should be evaluated annually in patients on deferoxamine. Testing of pulmonary function is also recommended, although not as frequently. Monitoring for chelator toxicity should also be followed as shown in Table 55-2.

## Summary

Iron overload is a significant cause of transfusion-related morbidity and mortality. With improved survival of patients with inherited or childhood transfusion-dependent anemias, older individuals with marrow failure, and cancer patients who have undergone intensive chemotherapeutic treatments or stem cell transplantation, the prevalence of this entity is likely to continue to grow. New technologies are now available for the noninvasive assessment of the body iron burden, making it easier to quantify the amount of excess storage iron in different tissues and to monitor the efficacy of chelation regimens. New oral drugs have made it easier for patients to be compliant with the chelation therapy that must be instituted in order to prevent tissue iron deposition and ameliorate the toxicity that would result from such deposition. As more information becomes available on the long-term use of deferasirox, combination therapy with deferoxamine may change the way chelation is prescribed. Research continues on the development of newer oral chelators. These advances hold promise for a new era of effective diagnosis, monitoring, and treatment of transfusional iron overload.

## Disclaimer

The author has disclosed no conflicts of interest.

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Acute noncardiogenic pulmonary edema occurring immediately after transfusion of donor plasma known to contain leucoagglutinins was first clearly described by Brittingham in 1957.<sup>1</sup> Scattered case reports of “allergic pulmonary edema” or “anaphylactic pulmonary edema” followed. In 1985 Popovsky and Moore<sup>2</sup> published the first prospective study of this complication that—recognizing the similarity in pathophysiology with the syndrome of acute lung injury (ALI) seen in critically ill patients—they called transfusion-related acute lung injury (TRALI). They reported a much higher incidence of the complication than had previously been considered likely, and following their report, increasing numbers of individual cases and small case series were published. However, with the development of hemovigilance schemes in the late 1990s, it became apparent that TRALI was not only an important cause of transfusion-related morbidity but, with the reduction in infective complications of transfusion, the leading cause of transfusion-related mortality. In the first decade of this century, recognition and understanding of the condition has further increased and the first serious efforts at prevention have been made.

## Clinical Features

Clinical reports of TRALI describe a sudden deterioration in lung function closely related to blood transfusion. The changes occur rapidly and generally begin within 2 hours and nearly always within 6 hours of the subsequently implicated transfusion. A small number of reports suggest that the onset may rarely be delayed until 12 hours or more after transfusion.<sup>3</sup> The conscious patient describes a tightness in the chest, feels short of breath, develops a dry cough, and may also experience nausea, dizziness, and rigors. On examination the patient is hypoxic, is often

hypotensive, and has tachypnea and tachycardia. Widespread crepitations are heard on auscultation of the chest. Rigors and fever are commonly reported but are not always present and fever may develop only some hours after the transfusion.

On intubation, or on suction of the already ventilated patient, a typical finding is of a copious frothy tracheal exudate, much like lightly whipped egg white. The nature and quantity of this exudate are often remarked on by attending anesthetists and it may be considered one of the hallmarks of severe TRALI. Arterial blood gases show hypoxia and hypercapnia that is often severe. Chest x-rays show nodular shadowing typically in the “bat’s wing” pattern of acute respiratory distress syndrome (ARDS) (Fig 56-1). Physiologic measurements such as pulmonary artery wedge pressure or esophageal Doppler study of the left atrium show systemic hypovolemia that may be marked. Further diagnostic tests of value are described below.

These cases represent the severe end of the spectrum of the disorder. Most early published reports describe severe cases such as these, but with more knowledge of the condition many milder cases are being recognized. Milder cases are proportionately less dramatic.

Recovery of respiratory function starts as early as 6 hours after the onset in milder cases but in severe cases deterioration may continue until 24 hours or beyond.

Some authors have reported a slightly different clinical picture in which the chief signs are rigors and fever with transient respiratory dysfunction, hypertension rather than hypotension, and usually occurring within 30 minutes of transfusion. Pulmonary edema is not always demonstrated radiologically and recovery is within 1 or 2 hours. These reactions have typically been associated with cellular blood components. It has been suggested that these cases may represent a different and non-antibody-related etiology (see below) from the “classical” severe cases.<sup>4</sup> However, a similar spectrum of mild to moderate reactions of this nature has also been documented with transfusion of plasma containing antibody to a neutrophil antigen, anti-HNA-2a.<sup>5</sup>

Acute lung injury with noncardiogenic pulmonary edema is common among critically ill patients and is generally

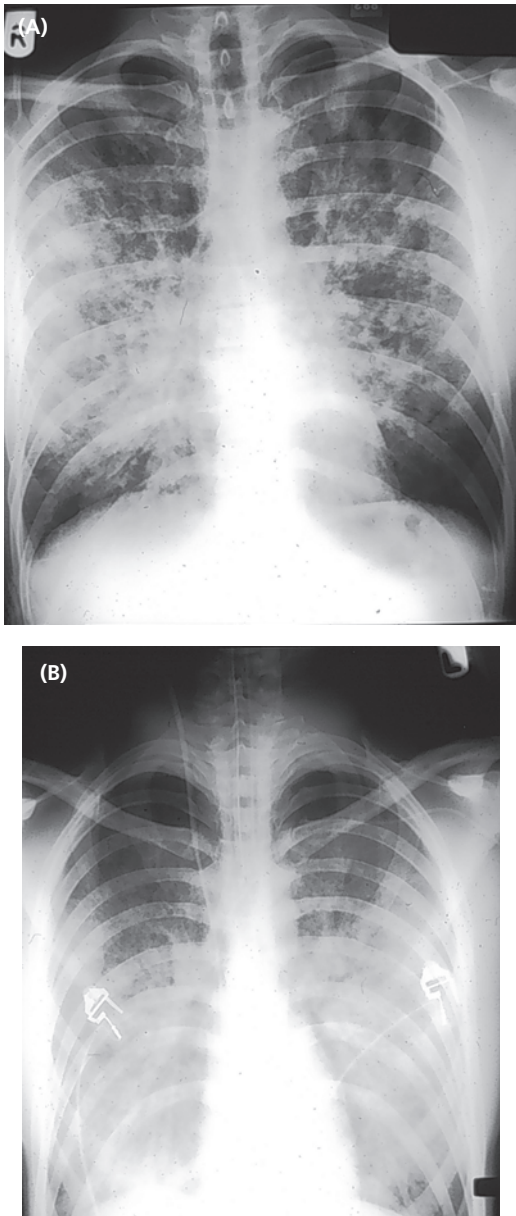


**Figure 56-1.** Chest x-rays from a 33-year-old man with severe TRALI, taken 2 hours (A) and 24 hours (B) after onset of symptoms, following transfusion of FFP containing HLA Class II antibodies. Note the “bat’s wing” pattern of edema with sparing of the lung bases, and the air bronchograms clearly visible on the first radiograph, and the more confluent airspace shadowing but still with basal sparing in the second x-ray.

considered to have a multifactorial pathogenesis. Many sick patients receive transfusion, especially after multiple trauma. There is evidence that TRALI is a significant contributor to ALI and that plasma-rich components from female donors are particularly implicated. The relationship between ALI and TRALI is further discussed below.

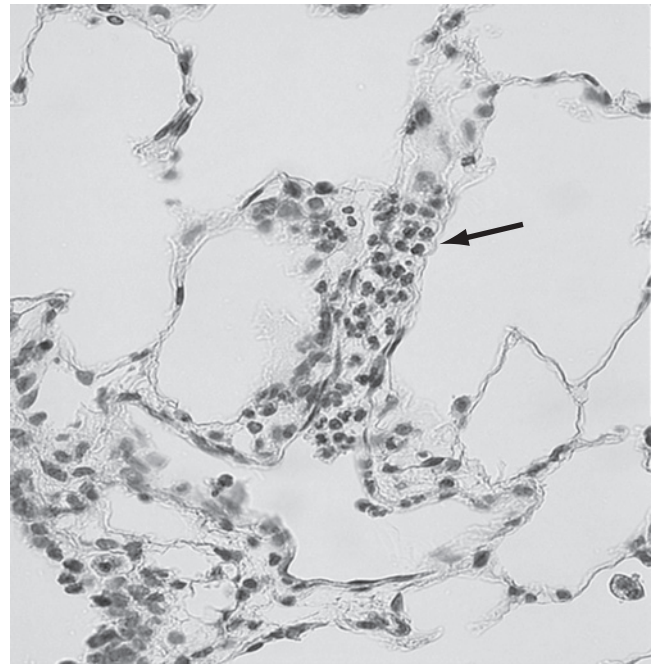


**Figure 56-2.** Sections of lung from a fatal case of TRALI. Note the presence of granulocytes in the capillaries (arrow indicates neutrophils).

## Pathophysiology

### Acute Lung Injury

ALI is the result of a capillary endothelial leak that allows fluid to pass from the pulmonary vessels, initially into the interstitial space and subsequently into the alveolar space. Because this edema is distinct from hydrostatic edema caused by cardiac failure or volume overload, it is sometimes known as nonhydrostatic edema. Numerous stimuli have been suggested to contribute to the likelihood of developing nonhydrostatic pulmonary edema including sepsis, trauma, aspiration of gastric contents, disseminated intravascular coagulation, and high tidal volume ventilation. In some cases of TRALI the transfusion appears to be the only probable cause of the lung injury, whereas in other cases it may be only one of several possible factors present. Histopathology, clinical findings, and experimental work have helped elucidate the nature of the stimulus from the transfused blood and the mechanism of the lung damage.

### Histopathology

Histopathologic findings from fatal TRALI cases are consistent with those for early ARDS, showing interstitial and intraalveolar edema<sup>2,3,6-10</sup> and extravasation of neutrophils into the interstitial and air spaces (Fig 56-2).<sup>3,6,7,10</sup> Hyaline membranes and destruction of the pulmonary architecture have been reported.<sup>6,7</sup> A consistent finding in TRALI is the presence of increased numbers of neutrophils within the pulmonary capillary vasculature and small pulmonary vessels.<sup>9,10</sup> On electron microscopic pictures, neutrophils were degranulated and focally in direct contact with

denuded stretches of the capillary wall. A positive correlation has been reported between capillary leukostasis and desquamated epithelial cells and between the degree of capillary leukostasis and the amount of proteinaceous fluid within the alveolar air spaces.<sup>10</sup> From these observations it appears that the neutrophil is central to the occurrence of lung damage. After sequestration in the early stages of TRALI, neutrophils and endothelial cells of the pulmonary microvasculature establish close contact. Activation of the neutrophils leads to endothelial damage and capillary leakage. The transit of proteinaceous fluid from the vessels into the air spaces results in acute pulmonary edema. In the later stage, especially of severe TRALI, neutrophils extravasate from the capillary into the alveoli and induce further pulmonary injury.

Evidence for an Antibody-Related Etiology

The relationship between TRALI and leukocyte antibodies in donor plasma was first noted by Brittingham, who reported that leukoagglutinins present in the plasma of a multitransfused patient induced an acute pulmonary reaction when transfused to a volunteer.<sup>1</sup> Severe pulmonary edema was similarly induced in a healthy volunteer who received an experimental gamma globulin concentrate, deliberately prepared from plasma that contained leukocyte, and, in particular, monocyte-reactive antibodies.<sup>11</sup> It is likely that this preparation contained high levels of HLA Class II antibodies. In addition to these cases of TRALI resulting from experimental transfusion of plasma containing leukocyte antibodies, there are numerous case reports of TRALI in which a transfused unit has been found to contain antibodies reactive with recipient leukocytes. In two large series of TRALI, where pulmonary infiltrates were apparent in chest x-rays, leukocyte antibodies in the donor of a transfused blood component were detected in 61% to 89% of cases.<sup>2,12</sup>

Animal models have provided confirmation of this antibody-mediated mechanism of TRALI. Severe vascular leakage was reproduced in isolated rabbit lungs by application of HNA-3a antibodies in an ex-vivo rabbit lung model.<sup>13</sup> From this experiment it was concluded that leukoagglutinating antibodies and concomitant complement activation are capable of causing TRALI. Other animal experiments have confirmed that antibodies are capable of causing TRALI but suggest that complement is not a prerequisite for TRALI induced by antibodies to CD177 (HNA-2a), because the induction of TRALI was found to be dependent on the density of the cognate antigen and occurred in a complement-free environment.<sup>14</sup> Alternative mechanisms leading to TRALI have been proposed and are discussed later, but the available evidence strongly suggests that donor leukocyte antibodies reacting with recipient antigens are the predominant mechanism.

Specificities of Antibodies Identified as Causing TRALI

Antibodies to HLA Class I and II antigens and to neutrophil antigens have all been clearly implicated as causing TRALI. Evidence from hemovigilance schemes and laboratories specializing in TRALI investigation has shown that the majority of cases (75-90%) are associated with HLA antibodies, and that with

Table 56-1. Leukocyte Antibodies Implicated in Transfusion-Related Acute Lung Injury\*

| Antigens     | Antibody Specificity |
|--------------|----------------------|
| HLA Class I  | A2                   |
|              | A9                   |
|              | A23                  |
|              | A24                  |
|              | A11                  |
|              | A28                  |
|              | A68                  |
|              | B7                   |
|              | B8                   |
|              | B12                  |
|              | B44                  |
|              | B45                  |
|              | B35                  |
|              | B57                  |
|              | B62                  |
| HLA Class II | DR1                  |
|              | DR4                  |
|              | DR6                  |
|              | DR7                  |
|              | DR8                  |
|              | DR9                  |
|              | DR14                 |
|              | DR17                 |
|              | DR51                 |
| HNA          | DR52                 |
|              | DR53                 |
|              | HNA-1a               |
|              | HNA-1b               |
|              | HNA-2a               |
|              | HNA-3a               |

\*Only antibody specificities that matched the antigens of the transfusion recipient are listed.

improved detection techniques about 50% of these are directed against Class II antigens.<sup>15</sup> Antibodies to neutrophil antigens are found in about 10% to 25% of cases. The spectrum of antibody specificities is shown in Table 56-1.

Mechanisms of Lung Damage in TRALI

Priming and Activation of Neutrophils

In TRALI the endothelial and alveolar cell damage appears to be mediated chiefly by activated neutrophils. Neutrophils activated by injurious agents will respond by the release of preformed granular enzymes and proteins and by the de-novo synthesis of a range of highly toxic reactive oxygen species (ROS). Neutrophils may be activated directly by one sufficiently strong stimulus, but the process often requires two or more stimuli. The first or “priming” stimulus will potentiate the response to a second or “activating” stimulus. A large percentage of patients who develop TRALI are sick, and there is in-vivo evidence that surgical

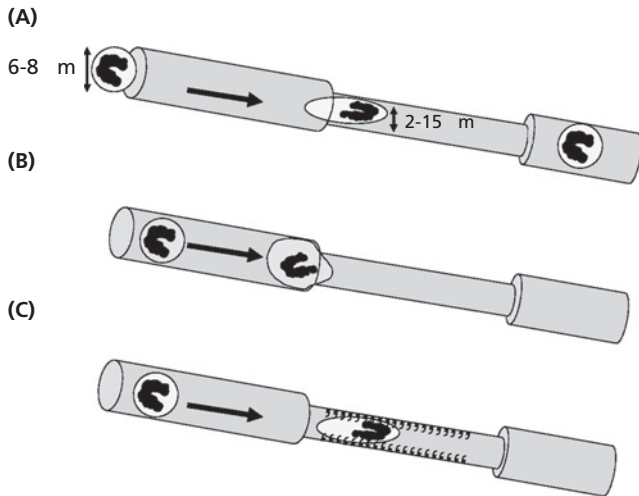


**Figure 56-3.** The neutrophil's passage through the pulmonary microvasculature. Approximately 50% of all pulmonary capillaries surrounding the alveoli have a smaller diameter than the spheric neutrophil. (A) In order to pass through the capillary, neutrophils are forced to pause, deform, and assume a "sausage shape" that allows transit of the capillary. (B) Neutrophil priming is associated with a decrease of deformability (called "stiffening"), which results in local trapping of the cell and a prolonged overall transit time. (C) Activation of the endothelial cells of the capillaries results in the upregulation of surface ligands, which also results in local trapping of the cell and a prolonged overall transit time.

procedures and active infections induce neutrophil priming.<sup>16-18</sup> In response to priming agents, neutrophils undergo polarization, a process that leads to "stiffening" of the cell.<sup>19</sup> This "stiffening" augments mechanical retention of neutrophils within the pulmonary capillary bed and prolongs their passage through the lungs (see below).<sup>20</sup> Prolonged, close contact between neutrophils and the endothelium provides a micro-environment in which transmembrane receptors and released mediators of each cell type can interact closely. Sequestered neutrophils, having been primed in the circulation, and endothelial cells can be activated by exogenous stimuli present in the blood bag. These transfused stimuli include antibodies, cytokines, and bioactive lipids (see below).<sup>21,22</sup>

Antibodies to neutrophil antigens involved in TRALI cases are able to prime and to activate neutrophils in some cases without additional stimuli,<sup>14,23-25</sup> explaining why even completely healthy individuals can develop TRALI if the antibody stimulus is sufficient.<sup>11</sup>

### Aspects of Neutrophil Passage through the Pulmonary Microvasculature

The alveolar capillary bed is a complex interconnecting network of short capillary segments. The path of a neutrophil from arteriole to venule crosses up to eight or more alveolar walls and encounters more than 50 capillary segments. Approximately half of these pulmonary capillaries are narrower than the diameter of a spherically shaped neutrophil (Fig 56-3). This forces neutrophils to slow and to deform before passing through the narrow capillary segment.<sup>26</sup> The transit time of neutrophils through the

pulmonary microvasculature is mainly affected by their deformation time, and slow transit accounts for significant accumulation of neutrophils in the lungs.<sup>27</sup> The pulmonary circulation normally contains about 28%, the "marginated pool," of the total blood neutrophil pool.<sup>28</sup> The stimulus-induced decrease in deformability appears to be more important than selectin-mediated rolling, a key mechanism of neutrophil adhesion within other capillary beds, but changes in surface receptors in primed neutrophils will also lead to molecular adhesion to endothelial cells.<sup>27,29,30</sup> Under physiologic conditions, primed and locally trapped neutrophils migrate from the capillaries into the alveoli as part of a local inflammatory reaction. In TRALI the primed and trapped neutrophil encounters a further activation signal in the form of transfused antibody or other transfusion stimulus, activates its microbicidal arsenal, and induces endothelial damage.

### Activation of Pulmonary Endothelial Cells

TRALI can also be triggered by activated pulmonary endothelium. In addition to constitutively expressed surface receptors, activated endothelial cells upregulate surface membrane receptors that facilitate neutrophil adhesion and priming.<sup>31-34</sup> Primary activation of endothelial cells has been suggested as the mechanism responsible for TRALI induction after infusion of bioactive lipids.<sup>35,36</sup>

### Neutrophil/Endothelial Cell Interplay

The interplay between neutrophils and endothelial cells, regardless of whether it has been started by neutrophil or endothelial cell activation, contributes largely to lung damage. Neutrophils respond to endothelial cell-derived mediators by activating and expressing integrins and by releasing proinflammatory mediators and granule contents. Released mediators activate endothelial cells, which, in turn, mobilize selectins, upregulate adhesion proteins, and produce inflammatory mediators; thereby, they enhance neutrophil adhesion and activation. It is within this interplay that the lung barrier breaks down and allows transit of proteinaceous fluid and, later, of neutrophils into the alveolar space. ROS released from neutrophils may play a relevant role in this process.<sup>7,14,37,38</sup>

### Mechanisms of Lung Injury by Different Mediators in Transfused Blood Components

The exact pathway leading to lung damage associated with transfusion depends on the nature of the antibody or other stimulus and the interplay between it and the cellular components.

### Antibodies to Human Neutrophil Antigens

Serologic work-up of TRALI patients identified antibodies to human neutrophil antigens in a number of cases.<sup>39-41</sup> In hemovigilance schemes, they are detected in approximately 10% of cases. As discussed above, the ability of these antibodies to induce TRALI has been shown in ex-vivo models of lung injury.<sup>13,14</sup> HNA antibodies, particularly those of HNA-2a, HNA-3a and HNA-4a specificity, are capable of directly activating neutrophils, which appears to be the mechanism by which they induce TRALI [Fig 56-4(A)].<sup>14,23,42,43</sup>

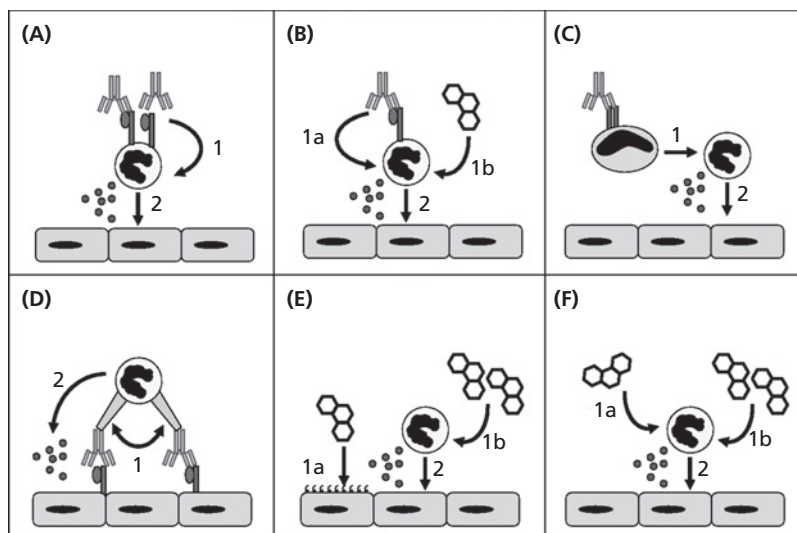


**Figure 56-4.** Proposed mechanisms of TRALI. The activation of neutrophils and the subsequent release of toxic agents that harm the endothelium is a key mechanism in TRALI. Epidemiological, clinical, and experimental data show that most TRALI reactions are induced by antibodies present in a transfused blood component. These antibodies may recognize epitopes on the surface of (A, B) the neutrophil, (C) the monocytes, or (D) endothelial cells. Binding of HNA or HLA Class I antibodies to neutrophils causes direct activation of the cell. Binding of antibodies, especially of HLA Class II antibodies, to monocytes has been proposed to induce the release of mediators that activate the neutrophil. Experimental data

demonstrate that neutrophils may also be activated indirectly when HLA Class I antibodies are bound to the endothelium of the lung, where they can recruit neutrophils via their Fc receptors binding to the Fc parts of these antibodies. Fc receptor cross-linking leads to neutrophil activation. (E-F) Biologically active substances other than antibodies (eg, bioactive lipids and CD40L derived from cellular blood components) are thought to cause some cases of TRALI. These substances are usually too weak to activate neutrophils directly, but may induce TRALI in concert with other factors that activate endothelial cells or neutrophils. See text for details.

### Antibodies to HLA Class I Antigens

Only a few studies have investigated the mechanisms by which major histocompatibility complex (MHC) Class I antibodies induce a TRALI reaction. An elegant study performed on mice by Looney and coworkers<sup>44</sup> presented in-vivo data on the mechanism of endothelial cell-dependent TRALI [Fig 56-4(D)]. Transfusion of an MHC Class I monoclonal antibody to mice expressing the cognate antigen induced TRALI and acute peripheral blood neutropenia. Mice lacking neutrophils and mice lacking the Fc $\gamma$ -receptor were resistant to MHC Class I antibody-induced TRALI. Transfer of wild-type neutrophils into FcR $\gamma^{-/-}$  mice restored TRALI following antibody infusion. This model is consistent with binding of the antibody directly to endothelial cells, in the first vascular bed encountered after injection, and recruitment of neutrophils through binding of the immunoglobulin Fc portion to the neutrophil Fc $\gamma$ -receptor. The protection observed in FcR $\gamma^{-/-}$  mice argues against direct neutrophil activation by the antibody. A clinical example of TRALI in keeping with such a mechanism was reported by Dykes et al<sup>45</sup> in a patient who had undergone lung transplantation several weeks earlier and who developed lung injury in the transplanted lung only following transfusion. An antibody to HLA-B44 (HLA Class I antigen) was present in the donor of one of the transfused units, and the cognate antigen was expressed on the transplanted lung but not on the patient's native tissues.

In this case direct Fab-dependent interaction between MHC Class I antibodies and the neutrophils could not have

contributed to the neutrophil recruitment and subsequent lung injury. However, proof that HLA Class I antibody may also cause TRALI by direct binding to neutrophil antigens comes from case reports of “inverse TRALI.” In one well-documented case infusion of human granulocytes caused severe lung injury in a patient who had Class I antibodies.<sup>50</sup> The antibodies cannot have reacted with the native endothelial cells but did react in vivo with the donor granulocytes, in keeping with a mechanism of TRALI by direct activation of neutrophils [Figs 56-4(A) and 4(B)].

In summary, cases of TRALI caused by HLA Class I antibody may be by specific antibody binding directly to endothelial cells with Fc-induced neutrophil aggregation, by specific antibody binding to leukocytes, or by a combination of both.

### Antibodies to HLA Class II Antigens

The biological mechanism by which HLA Class II antibodies induce TRALI remains to be fully elucidated. Corresponding Class II antigens are not expressed on resting human neutrophils, although they may be expressed upon neutrophil stimulation.<sup>46</sup> Expression of HLA Class II antigens on activated endothelium has been described, but HLA Class II antigen expression was not found on vascular endothelium of pulmonary capillaries or intravascular neutrophils in a patient who experienced fatal TRALI caused by an HLA Class II antibody.<sup>38</sup> Blood monocytes and intraalveolar macrophages normally express HLA Class II, and one possibility is that binding of antibodies to HLA Class



II causes release of cytokines from monocytes and activation of neutrophils within the pulmonary circulation [Fig 56-4(C)].<sup>47,48</sup> It is uncertain at present whether local monocytes are able to produce sufficient amounts of cytokines to significantly alter the activity of neutrophils and/or the endothelium.

It is unlikely that transfused antibodies have direct access to the alveolar space through an intact endothelium in sufficient concentration to induce release of cytokines and subsequent activation of neutrophils and/or endothelial cells, but where there is already some damage to the pulmonary endothelium, such a reaction may exacerbate ALI.

It remains possible that detection of antibodies to HNA and HLA could be surrogates for antibodies to yet unknown antigens on other cell types, eg, on monocytes. Alloantibodies to these or other cells might explain some apparently antibody-negative cases.

### Inverse TRALI: Transfusion of Neutrophils

In most cases of TRALI, antibodies or neutrophil-priming agents present in the blood component are causative for the pulmonary reaction. However TRALI, as described above, has also been reported in alloimmunized patients receiving blood components that contain neutrophils. Viable neutrophils may still be present in other blood components and Popovsky and Moore estimated that 6% of observed TRALI cases were caused by antibodies present in the recipient.<sup>2</sup> As universal leukocyte reduction is introduced in more countries, inverse TRALI caused by leukocytes in platelet concentrates (PCs) and Red Blood Cells (RBCs) will become less important, but will remain of particular relevance to patients receiving granulocyte transfusions.<sup>49,50</sup>

### Bioactive Lipids

Blood components may accumulate intermediate metabolic products, such as bioactive lipids, during storage. These substances are breakdown products of membrane lipids, including lyso-phosphatidylcholines [C16, C18 lyso-platelet activating factor (PAF)], and act on neutrophils through the cells' PAF receptors in order to prime the respiratory burst reaction.<sup>51</sup> Because these neutrophil-priming agents do not develop in stored acellular plasma, their generation is dependent on the presence of blood cells. It has been demonstrated that posttransfusion sera from these patients contained significantly more neutrophil-priming activity than the controls,<sup>7</sup> and were also reported to induce TRALI after the transfusion of stored autologous blood.<sup>52</sup> In an ex-vivo rat lung model of TRALI, addition of both lipopolysaccharide (LPS) and plasma containing neutrophil-priming lipids obtained from stored RBCs was necessary to induce TRALI,<sup>7</sup> which is in accordance with the pathophysiologic model that priming precedes efficient activation of neutrophils [Fig 56-4(E) and (F)]. Plasma from stored PCs and containing neutrophil-priming lipids can cause TRALI in an identical animal model.<sup>36</sup> In this model, rats were treated with LPS to approximate active infection. Lungs from LPS-treated animals perfused ex vivo with plasma from stored RBCs or stored PCs, but not plasma from identical fresh RBCs or PCs, evidenced

TRALI.<sup>51</sup> Lungs pretreated with vehicle instead of LPS did not evidence TRALI with any of the perfusates.

### CD40-Ligand

CD40 ligand (CD40L) is another neutrophil-priming breakdown product. It is a platelet-derived proinflammatory mediator found in cell-associated and soluble (sCD40L) forms. It may be found in PCs and accumulates during storage.<sup>53</sup> sCD40L binds to CD40, which is present on the surface of monocytes, macrophages, and neutrophils.<sup>54</sup> This CD40L/CD40 interaction induces neutrophil priming. CD40L has been identified as a possible cofactor in TRALI because its concentration in transfused PCs that were involved in TRALI cases was found to be significantly higher than in control units. In vitro, human microvascular endothelial cells preincubated with LPS experienced severe damage when sCD40L-primed neutrophils were added, whereas unprimed neutrophils did not induce such damage.<sup>54</sup>

### Immune Complexes

Incubation of neutrophils with immune complexes (ICs) results in production of tumor necrosis factor alpha (TNF- $\alpha$ ) and induced apoptosis of endothelial cells in vitro.<sup>55</sup> It remains speculative whether antibodies present in blood components might form ICs with their corresponding soluble HNA or HLA antigens in the recipient's circulation and so prime or activate neutrophils in the pulmonary circulation.

### Immunoglobulins

Normal IgG has been postulated to activate neutrophils in a patient with osteopetrosis being treated with gamma interferon and granulocyte and monocyte colony-stimulating factor.<sup>56</sup> The patient had very low levels of endogenous IgG1 and IgG2 and developed severe lung injury shortly after transfusion of platelets from an untransfused male donor. No leukocyte antibodies could be found either in donor or recipient. It is suggested that transfused IgG binding to the neutrophils, which were already primed by interferon and stimulating factor, was sufficient to cause neutrophil activation and lung injury. This case may be considered to be a good example of "multiple hit" TRALI.

Reports of lung injury following intravenous IgG infusion are rare and seem to be associated with high doses or concentrates prepared intentionally with high level of leukocyte antibodies.<sup>11,57</sup> It is possible that antibodies are both diluted and neutralized during the preparation of the pooled product as suggested for pooled viridically treated plasma.<sup>58,59</sup>

### Multiple Hit/Threshold Theory of TRALI Causation

Where the stimulus to endothelial and neutrophil activation is sufficient, lung damage can occur in an otherwise healthy individual with no other likely cause of lung injury. Evidence for this comes from reports of TRALI in transfused volunteers as described above and also from reports where plasma has been used for clinical reasons in otherwise healthy individuals. These cases are a minority and most patients receiving transfusion and

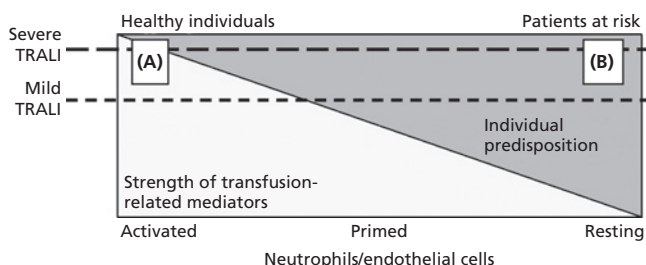


**Figure 56-5.** A TRALI threshold model. The TRALI threshold model proposes that a certain threshold must be overcome to commence a TRALI reaction. The threshold of mild TRALI, in which oxygen supply is sufficient, is lower than that of severe TRALI, in which patients require mechanical ventilation (horizontal lines). In order to overcome this threshold, numerous factors must act in concert. These factors can be summarized as strength of exogenous transfusion-related mediators (light box) and individual predisposition of the patient (grey box). The individual predisposition covers both constitutive (genetic) factors and dynamic or acute influences, ie, acute infection or trauma. A strong exogenous transfusion-related mediator, such as a strong activatory neutrophil-specific antibody, will precipitate a TRALI reaction even if the influence of the individual predisposition is low (example A, in an otherwise healthy recipient). In contrast (example B, an individual “at risk,” such as a septic patient with an activated pulmonary endothelium), a relatively mild exogenous transfusion-related mediator with low neutrophil-priming activity will be sufficient to overcome the threshold. Used with permission from Bux and Sachs.<sup>60</sup>

especially transfusion of plasma have significant comorbidities, some or many of which may also result in priming or activation of neutrophils or damage to pulmonary endothelial cells. It has been suggested that TRALI will be more common in such patients. Early reports noted that most patients with TRALI had recently undergone surgery and suggested that this in itself was a sufficient second stimulus in many cases. Some experimental evidence for the theory of “two hit” or “multiple hit” theory has been provided by studies on bioactive lipids described above.<sup>36</sup> The theory of “multiple hits” has been further developed by Bux and Sachs,<sup>60</sup> who suggest that the neutrophil is central to the pathogenesis of TRALI and that activation of the neutrophil requires sufficient stimuli from one or more sources to reach a certain threshold at which point full activation and lung damage will ensue (Fig 56-5). Depending on the magnitude of the neutrophil response, the lung damage can be mild or severe with corresponding clinical effects.

### ALI and Transfusion in Critically Ill Patients

Clinical evidence for the theory that “multiple hits” may result in TRALI has been provided by studies involving critically ill patients who are known to be susceptible to ALI. Numerous retrospective studies have suggested a relationship between the amount of blood transfused and morbidity and mortality. All these studies are beset by the difficulty in allowing for the confounding factor of how much blood the patient required also being a marker for the severity of the illness. The Transfusion Requirements in Critical Care (TRICC) trial of transfusion triggers was both prospective and randomized and found a

significantly higher incidence of pulmonary edema and a higher incidence of ALI in patients receiving more transfusion where the standard component was relatively plasma rich.<sup>61</sup> Gajic et al<sup>62</sup> studied the clinical associations with ALI developing in patients during mechanical ventilation that was required for management of critical illness. They found a strong association with transfusion of plasma, but not with red cell transfusion, age of transfused red cells, the leukocyte content of transfused red cells, or with platelet transfusion. Further studies identified transfusion of female plasma as being particularly associated with development of ALI in keeping with an antibody type of mechanism.<sup>63</sup> A prospective study in intensive care patients comparing male donor and parous female plasma found similar results.<sup>64</sup> The incidence of ALI associated with transfusion in these studies was of the order of 1 in 50 to 200 units of female plasma transfused, a far higher incidence than the reported incidence of TRALI in other circumstances. These findings are in keeping with the “multiple hits” or “threshold” model of TRALI in which highly susceptible patients subject to multiple toxic insults to neutrophils and endothelial cells develop lung damage after a relatively mild additional stimulus from transfusion.

## Diagnosis and Differential Diagnosis

### Respiratory Dysfunction

The development of new respiratory dysfunction caused by pulmonary edema in association with recent transfusion should be considered as evidence of possible TRALI. Significant respiratory dysfunction consistent with ALI can be defined as a decrease in transcutaneous oxygen saturation to less than 90% or an arterial  $pO_2$  of less than 60 mm Hg while breathing room air, or a  $PaO_2/FiO_2$  ratio of less than 300 mm Hg.

Pulmonary edema can be demonstrated by clinical examination and chest x-ray. Alternative causes of sudden respiratory dysfunction, without edema, include transfusion-related problems such as allergic reactions with bronchospasm, shock associated with a bacterially infected unit or with ABO incompatibility, and causes unrelated to the transfusion such as cardiac arrhythmias, infection, and pulmonary embolus (Fig 56-6).

### Distinguishing between Hydrostatic and Nonhydrostatic Pulmonary Edema

Once pulmonary edema is demonstrated, it is necessary to determine whether it is cardiogenic (hydrostatic) or caused by increased capillary permeability as in TRALI and other forms of ALI (nonhydrostatic). Cardiogenic pulmonary edema may be caused by transfusion-associated cardiac overload (TACO) or may be caused by factors unrelated to transfusion, such as simple overhydration, especially in renal failure.

### Radiology

Radiographic appearances of edema from increased pulmonary capillary permeability are often characteristic with patchy



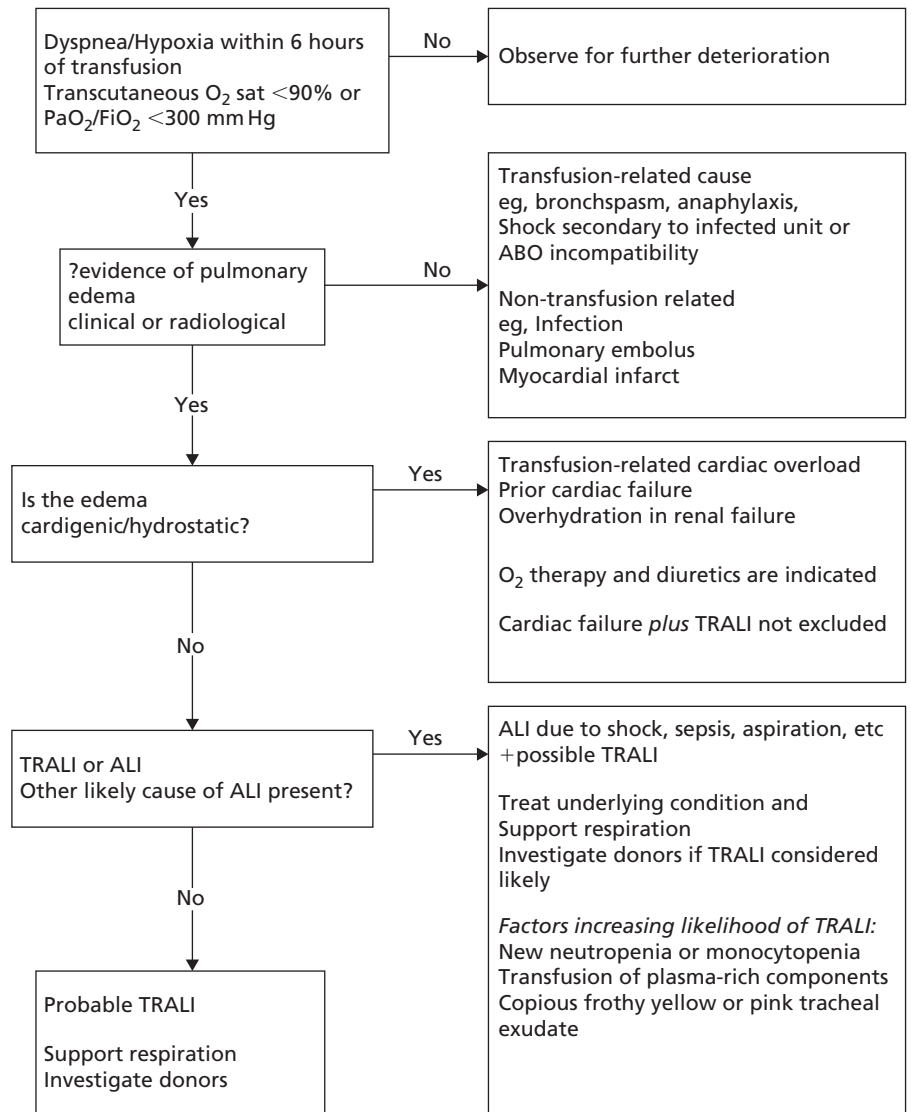


**Figure 56-6.** Flow chart for diagnosis of TRALI.

or nodular shadowing, mainly peripheral but sparing both the apices and the costophrenic angles and with the appearance of air bronchograms. This pattern is sometimes likened to a bat's wing. In contrast, cardiogenic edema typically shows upper lobe venous distension and edema in the perihilar and basal areas. Edema in overhydration or renal failure is typically perihilar, and shows no air bronchograms. In severe TRALI or in later stages, the radiologic appearances may progress to a complete white-out of the lung fields.

### Physiologic Measurements

Physiologic measurements are aimed primarily at assessing the cardiac status. Measurement of the left atrial pressure or volume may be by pulmonary artery wedge pressure through a Swan-Ganz catheter, via esophageal Doppler ultrasound, or by transthoracic echocardiography. High left atrial filling pressure or volume suggest cardiogenic pulmonary edema or fluid overload.

Normal or low pressure or volume is in keeping with noncardiogenic pulmonary edema. Low levels indicate hypovolemia, a common finding in TRALI. Electrocardiography may also be helpful in detecting cardiac strain patterns or evidence of infarction.

Prior or new cardiac failure does not exclude the possibility of TRALI, because both may be present in the same patient. However, cardiac failure does indicate that the use of diuretics, which are otherwise contraindicated in TRALI, may be beneficial.

### Laboratory Tests

A serum level of B-type natriuretic peptide (BNP) of less than 250 pg/mL is consistent with ALI rather than cardiac failure, while a level of greater than 250 pg/mL or a twofold increase from a previous level is consistent with cardiac failure.<sup>65</sup> A low level is not completely specific for ALI nor very sensitive, as many patients with ALI also have cardiac dysfunction. A high level may also be seen in renal failure.

**Table 56-2.** 2004 Consensus Conference Definition of TRALI<sup>66</sup>

|                                                                        |
|------------------------------------------------------------------------|
| I. TRALI criteria                                                      |
| A. ALI                                                                 |
| 1. Acute onset                                                         |
| 2. Hypoxemia                                                           |
| a. Research setting:                                                   |
| 1) $\text{PaO}_2/\text{FiO}_2 \leq 300$ , or                           |
| 2) $\text{SpO}_2 < 90\%$ on room air                                   |
| b. Nonresearch setting:                                                |
| 1) $\text{PaO}_2/\text{FiO}_2 \leq 300$ , or                           |
| 2) $\text{SpO}_2 < 90\%$ on room air, or                               |
| 3) other clinical evidence of hypoxemia                                |
| 3. Bilateral infiltrates on frontal chest radiograph                   |
| 4. No evidence of left atrial hypertension (ie, circulatory overload)  |
| B. No preexisting ALI before transfusion                               |
| C. During or within 6 hours of transfusion                             |
| D. No temporal relationship to an alternative risk factor for ALI      |
| II. Possible TRALI                                                     |
| A. ALI                                                                 |
| B. No preexisting ALI before transfusion                               |
| C. During or within 6 hours of transfusion                             |
| D. A clear temporal relationship to an alternative risk factor for ALI |

A protein concentration in the pulmonary edema of greater than 70% of the serum protein is strong evidence in favor of a capillary to alveolar leak as seen in ALI from any cause.

### Implicating Transfusion as a Cause of Nonhydrostatic Edema

#### Consensus Definition of TRALI

A consensus definition of TRALI was agreed upon in 2004.<sup>66</sup> A clinical diagnosis of TRALI is considered where a new episode of ALI has occurred within 6 hours of a blood component or derivative transfusion. Where no other cause of ALI is found, the diagnosis of TRALI is considered probable whatever further tests show. Where another possible cause of ALI is present, the diagnosis of TRALI can only be considered possible (Table 56-2). These criteria are useful but do not include laboratory tests. Many cases of TRALI will be in patients with competing etiologies for ALI. Further recipient and donor investigation can help determine, often in retrospect, the probability that the lung injury was wholly or partly due to transfusion.

#### Laboratory Tests

##### *Changes in Circulating Leukocytes*

Leukopenia and, in particular, neutropenia and monocytopenia occurring within the first hour after the transfusion and possibly present before the development of clinical lung injury, are supportive of transfusion as opposed to other causes of ALI. The neutropenia is often followed by neutrophilia 5 to 6 hours later.<sup>1,67</sup> Monocytopenia is common, may be absolute, and may be more persistent than the neutropenia.<sup>67,68</sup> Neither change is completely specific or sensitive. The degree of neutropenia is likely to be greater with neutrophil-specific antibodies such as

anti-HNA-2a, and with neutrophil agglutinating antibodies such as anti-HNA-3a and anti-HLA-A2, whereas monocytopenia alone is most likely to be seen with HLA Class II antibodies.<sup>68</sup> Neutropenia is seen without evidence of lung damage with some neutrophil-specific antibodies.<sup>5,69</sup>

#### Donor Tests

To implicate a particular donation as a cause of antibody-related TRALI requires leukocyte typing of the recipient and antibody testing of the donors (see below). Samples for neutrophil and HLA typing should be taken early from the patient in case death ensues. The finding of a donor/recipient antibody/antigen match is of value in donor management and will make the diagnosis of TRALI more likely than a negative investigation, especially where only a small number of units have been transfused. Such matches also occur in patients without TRALI, and are of increasing statistical likelihood where a large number of units have been transfused. A positive match does not prove that TRALI has occurred.

### Management and Outcome

#### Management of the Patient

The mainstay of treatment is respiratory support, either with oxygen alone, or with oxygen and continuous positive airway pressure by mask or mechanical ventilation. In severe cases with systemic hypovolemia, there must be adequate fluid replacement in addition to positive pressure ventilation. This will often require central venous pressure or left atrial volume monitoring. Additional strategies such as high-frequency ventilation and prone ventilation can be of value. Diuretics are contraindicated, because they have been clearly reported to worsen the hypovolemia and hypotension in several cases.<sup>49,70</sup> Only where there is concomitant fluid overload from cardiac failure or other causes are diuretics indicated. The use of corticosteroids after the insult is of uncertain value. Where the diagnosis is made within 6 hours and the lung damage is severe, use of high-dose steroids might in theory limit further damage by inhibition of neutrophil activation. Prednisolone, methylprednisolone, and dexamethasone have all been used, but there is no useful clinical evidence regarding efficacy. Where the diagnosis is made later, they are unlikely to be of value. In intractable cases for which maximal ventilation is insufficient, extracorporeal membrane oxygenation has been used to support the patient until recovery.<sup>71</sup> The use of plasmapheresis with the intent of removing the causative antibody has been reported.<sup>11</sup> Further transfusions should be given if clinically indicated, but plasma-rich products from female donors should be avoided.

#### Clinical Course and Outcome

Most patients begin to improve by 24 hours after the initial injury. Milder cases improve as early as 6 hours. Chest x-rays usually show clearance of edema by 48 to 96 hours. Nearly all patients who recover do so without any long-term lung damage. Other organ damage such as acute renal failure is seen in more

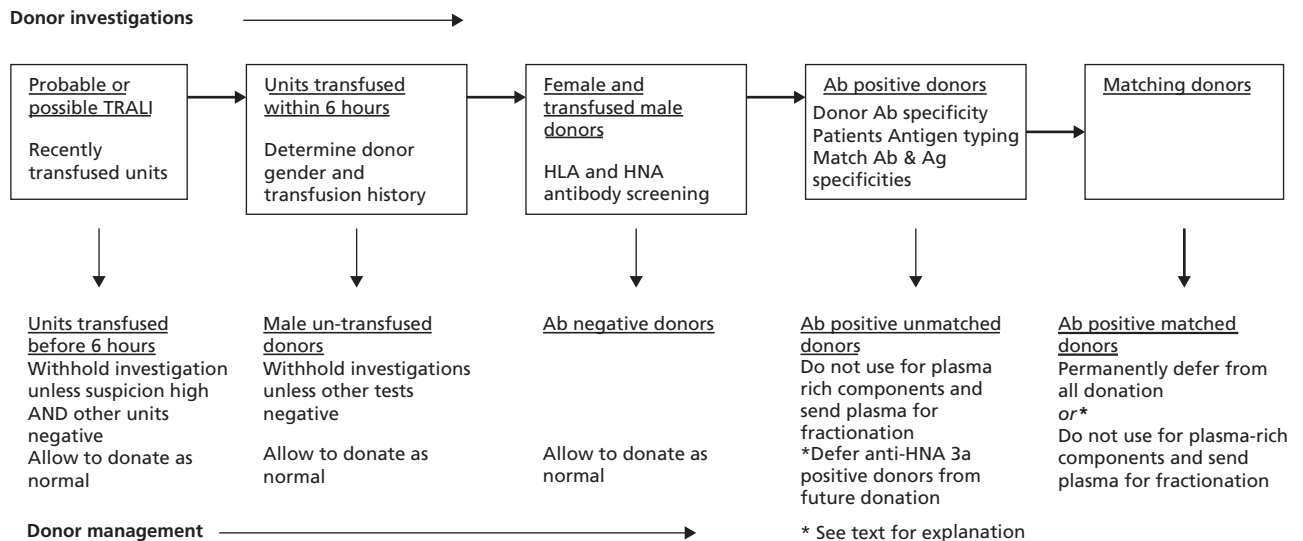


**Figure 56-7.** Flow chart for investigation and management of donors.

severe cases, although it is not clear whether this is all due to hypoxia and hypovolemia or whether there is damage to capillary beds in organs other than the lungs.<sup>67</sup> Mortality in different published series varies between 5% and 30%. This depends in part on how cases are ascertained. Where patients receiving transfusion were assessed prospectively for the complication, the incidence was higher, presumably because of recognition of milder cases, but the mortality was low (6%). Where reports depend on a physician recognizing and reporting the condition, the incidence is lower but mortality higher. As an example, the hemovigilance scheme in the United Kingdom (UK) reported seven deaths out of 28 possible, probable, or likely cases reported in 2002, a mortality of 25%.<sup>72</sup> Mortality is more likely in patients receiving larger volumes of single donor plasma [including Fresh Frozen Plasma (FFP)] rather than RBCs and more likely in patients with more comorbidities.

### TRALI in Neonates and Children

TRALI has been reported in children with the same features as noted in adults, including leukopenia, nonhydrostatic pulmonary edema, and hypovolemia. Of 12 published reports, four ascribed death of the child partly or wholly due to the transfusion reaction.<sup>56,73,74</sup> Age range of cases is from 0 months to 16 years but only two neonatal cases have been reported,<sup>49,75</sup> and no neonatal cases have been reported to the UK hemovigilance scheme over 10 years. Use of blood components is more common in neonates than older children, and neonates also receive larger volumes of single donor plasma relative to their weight. It is possible that neonates are in some way less susceptible to TRALI.

### Directed Donations and TRALI

A particular feature of pediatric TRALI is its occurrence after the use of directed donations from the mother. This practice

has been documented to cause TRALI and can be expected to be high risk by virtue of the high probability of the mother having leukocyte antibodies that will match her child's antigen specificity.<sup>76</sup> By the same rationale, directed donations from a wife to a husband, or of a leukocyte-containing component from a child to a mother or a husband to a wife, will be particularly likely to cause TRALI.

### Donor Investigation

Strategies of donor investigation vary among blood centers. The dual aim of investigation is to help establish the diagnosis and to allow appropriate management of implicated donors. A typical scheme adapted from Su and Kamel<sup>77</sup> is shown in Fig 56-7. Investigation of donors is undertaken only when a diagnosis of TRALI is considered probable or possible. Donations transfused within the 6 hours before the development of lung injury are considered to be under suspicion. Where the number of possibly implicated donors is small (four or fewer) all donors should be investigated. Where the number of possibly implicated donors is greater, the high-risk donors with a history of pregnancy or transfusion are initially investigated. If a donor or donors are found with an antibody that matches a cognate antigen, investigations of the lower risk donors are not undertaken. If no donor is implicated in the initial investigation, further lower risk donors can be tested in those cases that are considered to be probable TRALI. A further selection policy is to initially investigate only those donors of plasma-rich components. Investigations are undertaken at a qualified laboratory with adequate sensitivity for HLA Class I and II antibodies, and HNA antibodies. Recipient HLA and HNA typing may be performed prospectively or only when a donor antibody is found. Tests for recipient antibodies may be undertaken when the lung injury followed transfusion of granulocyte concentrate, or following non-leukocyte-reduced

component transfusion in which no donor/recipient antibody/antigen match can be identified. When a nonantibody mechanism is suspected, or if an antibody-related etiology has been largely excluded, tests on the residual donation for abnormal lipids or other bioactive substances may be carried out at a research or reference center.

### Management of Implicated Donors

Management of implicated donors varies among countries and centers. It is possible to permanently defer all donors who are implicated in a case of TRALI by virtue of having leukocyte antibodies of any specificity. This would undoubtedly exclude many donors unnecessarily and the logical extension of such a policy is that all donors with leukocyte antibodies should be deferred whether or not they have been possibly implicated in a case of TRALI. Alternatively, only those donors with antibody matching a recipient antigen are excluded from future donation. This policy assumes that some feature of the antibody in that donor makes them more likely to have caused TRALI in the index case, and thus to cause TRALI with further donations. Finally, it is possible to defer donors who are either possibly or definitely implicated only from donation of plasma-rich components. In addition, some centers automatically defer permanently any donor found to have anti-HNA-3a because of the high frequency of the antigen in the recipient pool (95%) and the common association with severe TRALI. As more countries restrict the manufacture, of plasma-rich components to male or nulliparous untransfused females, the policy of donor management will become less contentious.

## Incidence and Epidemiology

### Variables Affecting the Incidence of TRALI

The frequency of reported TRALI depends upon variables affecting the donors, the recipients, and the physicians. Antibody-associated TRALI, which is the common and severe form, is nearly always associated with blood donations from female donors with a history of pregnancy and is both more common and more severe with transfusion of plasma-rich products, such as FFP or apheresis platelets. HLA antibodies have been found in approximately 15% of all female donors but are more common in those who have had more pregnancies and more recent pregnancies,<sup>78,79</sup> and are even more common with more sensitive tests.<sup>80</sup> Human neutrophil antibodies are less common. Therefore, incidence of TRALI will depend in part on the donors used by the local blood center. Policies to reduce the number of components containing antibodies are discussed below.

Look-back studies for regular donors of blood whose antibody-containing plasma has been implicated in a case of TRALI show that many cases of TRALI are either overlooked or unreported. In addition, infusion of an antibody to a recipient with a matching antigen does not necessarily result in TRALI.<sup>81,82</sup> Both inherited and acquired recipient variables and details of

the transfusion will affect the likelihood of developing clinically apparent TRALI. Inherited variables have not been defined but will almost certainly include polymorphisms in the immune response pathways. The presence of high levels of soluble HLA antigens in the recipient's plasma may be protective.<sup>83</sup> Acquired recipient variables include the presence of other lung insults, and other forms of comorbidity as discussed earlier. Transfusion variables include the titer and specificity of antibody, the volume of plasma infused, and the speed of infusion. Plasma-reduced RBCs transfused over 90 minutes rarely cause TRALI, whereas FFP given over 15 minutes—a more than 50-fold higher rate of plasma transfusion—is not uncommonly implicated.

Finally, the likelihood of diagnosing and reporting TRALI depends heavily on the physician's knowledge of the condition.

### Reported Frequency of TRALI

One single hospital prospective study estimated frequency for all blood components transfused without any donor selection as 1 in 5000 units.<sup>2</sup> A retrospective single hospital study estimated the incidence as 1 in 7900 units of FFP only.<sup>67</sup> Hemovigilance data from voluntary reports in the UK found a reported incidence of approximately 1 in 30,000 units of FFP transfused.<sup>72</sup> Retrospective studies of ALI/TRALI in critically ill patients and a single prospective trial of TRALI after FFP infusion found that as many as 1 in 50 to 200 units of plasma from parous female donors could cause some respiratory dysfunction when compared to plasma from male donors.<sup>64,65</sup>

It is not possible to give a single figure for incidence, but the chance of female donor plasma containing an antibody matching a recipient antigen is greater than 1 in 50. Severe and clinically distinct TRALI probably occurs about 1 in 2500 to 4000 units of female donor plasma transfused. ALI in which transfusion is a contributory factor in a critically ill patient may be much more common.

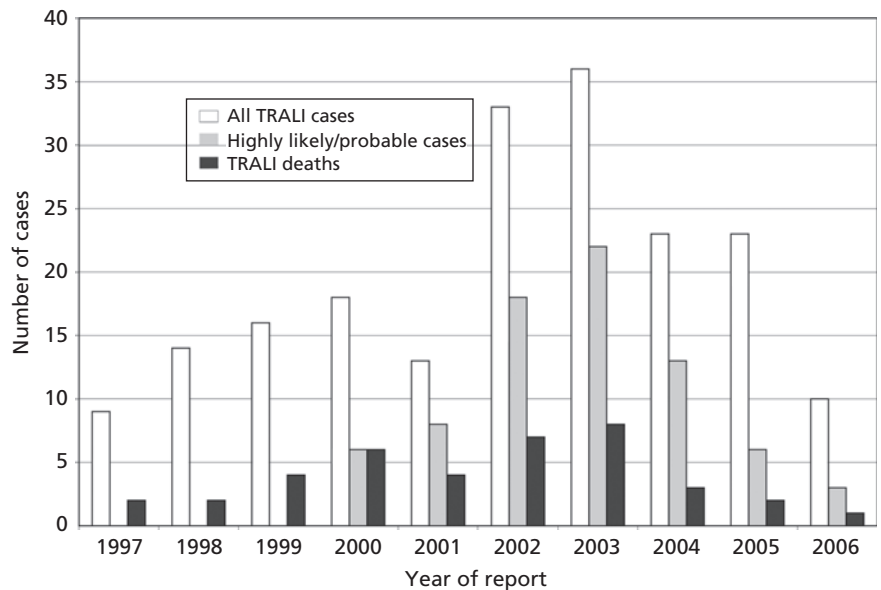
## Prevention

The majority of severe TRALI cases have an antibody-related etiology. Therefore, prevention is largely aimed at reducing the likelihood of transfusion of the causative antibodies. There are several strategies to be considered.

### Avoidance of Unnecessary Transfusion of Plasma

This includes unjustified transfusion of plasma components such as FFP and of unnecessary transfusion of plasma associated with RBCs. Use of FFP varies considerably among countries and it has been demonstrated that use to correct minor coagulopathies is both ineffective and unnecessary.<sup>84,85</sup> Avoidance of unnecessary transfusion of FFP will reduce the potential for transfusion of units containing antibody. RBCs in additive solution containing less than 20 mL residual plasma are uncommon causes of TRALI, and in those recorded cases there have usually been multiple antibodies in the donor plasma matching cognate antigens in the recipient. Whole blood donations, particularly

**Figure 56-8.** Cases of TRALI reported to the UK hemovigilance scheme from 1996 to 2006. After 1998 all cases were considered by an expert panel and classified as highly likely, probable, possible, or unlikely. From 1996 to 2003 case ascertainment rose steadily with increasing knowledge of the condition among physicians. From October 2003 a policy to procure FFP from male donors only was introduced. Following the change there was a decrease in reports, and a marked decline in highly likely or probable TRALI and in deaths from TRALI (note that cases were recorded by the year that the report was made, and that many cases documented for 2004 occurred in 2003). Data kindly provided by Dr. C Chapman on behalf of SHOT UK.



from high-risk donors (parous female or previously transfused donors), can be processed into RBCs in additive solution with minimal remaining plasma. Similarly, use of platelet additive solutions will reduce the volume of plasma with platelets.

### High-Risk Donor Exclusion

The most straightforward form of this policy is to exclude donations from all female donors from production of plasma-rich components. Some individual blood centers have followed such policies locally for some years.<sup>86</sup> The effectiveness of this intervention was demonstrated in the UK, where such a policy was instituted nationally in 2003. An active hemovigilance scheme clearly showed a 66% decrease both in the incidence of probable TRALI and in associated deaths over the next 3 years (Fig 56-8).<sup>87</sup> Further developments of this policy include excluding only females with a history of pregnancy and excluding any donor with a history of transfusion or organ grafting.

### Antibody Testing

By testing donors for HLA and HNA antibodies it is possible to exclude all donors with detectable antibodies, or those with antibodies of certain specificities or titer, either from all blood donation or just from donation of apheresis plasma-rich components. This strategy is attractive in that unnecessary exclusion of valuable donors is avoided but it also depends on the sensitivity and specificity of the antibody tests. It has been used with apparent success in some centers.<sup>88</sup>

### Use of Pooled Plasma

Pooled solvent/detergent-treated plasma does not appear to cause TRALI. It is hypothesized that this is because of dilution of antibodies, neutralization of HLA antibodies by soluble HLA antigens in the plasma of other donors, and subsequent removal of the immune

complexes in the processing.<sup>58,59</sup> Extensive use of solvent/detergent-treated plasma in Europe has not been associated with TRALI.<sup>89,90</sup>

### Nonantibody TRALI and Inverse TRALI

Leukocyte reduction reduces the production of cellular activation or breakdown products that have been implicated in TRALI. It also will prevent the rare but distinct cases of inverse TRALI related to bystander granulocyte transfusion.

## Conclusion

It has taken over 50 years for the full extent of TRALI to be appreciated. It is now recognized as one of the major adverse outcomes of transfusion. Preventive strategies are effective but risk loss of donors. These strategies will be refined over the next decade as more about the condition is learned.

## Disclaimer

The authors have disclosed no conflicts of interest.

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# V Cell and Tissue Therapy

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The human major histocompatibility complex (MHC) resides on the short arm of chromosome 6. The complete genomic sequence of this region contains more than 200 genes and spans 4 to 8 megabases, depending on where the telomeric boundary is drawn.<sup>1</sup> Many of the genes in the MHC encode proteins involved in immune responses, including the Class I and Class II antigenic molecules of the HLA system; the Class III molecules comprising complement components C2 and C4, and tumor necrosis factor; and the peptide transporter proteins TAP1 and TAP2. This chapter focuses on the genetic structure of the MHC, current HLA nomenclature, tests for detecting HLA, and the biomedical significance of HLA antigens.

The existence of white cell antigens independent of red cell genetic systems was suggested as early as 1952. In 1958, Jan Dausset<sup>2,3</sup> detected, in a patient who had undergone multiple transfusions, the first leukocyte antibody [called Mac (HLA-A2)]. This discovery was followed in 1958 by independent studies on similar antibodies in postpartum serum, reported by van Rood et al<sup>4</sup> and Payne and Rolfs.<sup>5</sup> These antibodies originally were thought to be autoimmune in nature or to be isoantibodies responsible for febrile (nonhemolytic) transfusion reactions. Early in 1960, Brunning et al<sup>6</sup> found that these antibodies could be used to detect several diallelic genetic systems present on the cells of most tissues. Until 1964, advances in HLA typing were hampered by inconsistent and nonspecific leukoagglutination testing methods. This status changed with the development of the microlymphocytotoxicity assay by Terasaki and McClelland.<sup>7,8</sup> In a slightly modified form, this test continued to be the standard for clinical HLA typing until the widespread adoption of nucleic-acid-based methods in the 1990s.

In 1964, the first International Histocompatibility Workshop was held. Since then, 13 workshops have been held at 2- to 4-year intervals. These workshops are designed to confirm scientific

findings, to develop unifying concepts regarding the MHC, and to upgrade existing HLA nomenclature. Table 57-1 presents an overview of the workshops, the year and their major themes. In the third workshop (1967), it was established that the HLA antigens belonged to the same genetic system,<sup>9</sup> and the term HL-A, coined from the Hu leukocyte system of Dausset et al<sup>10</sup> and the LA system of Payne et al,<sup>11</sup> was assigned to identify this system. By 1970, it was established through skin grafting studies with human subjects, particularly families with HLA-identical siblings, that these antigens were important in organ transplantation.<sup>12</sup> In 1973, it became clear from results of one-way mixed leukocyte cultures with homozygous testing cells that other genetic polymorphisms existed within the MHC.<sup>13</sup> Also by 1973, the remarkable association of HLA with certain diseases became apparent.<sup>14,15</sup> In the late 1970s, HLA-D (defined by mixed leukocyte culture) antigens were established,<sup>16</sup> serologically detected antigens on B lymphocytes [HLA-DR (D-related)] were found to be closely related to the HLA-D antigenic products,<sup>17,18</sup> and the existence of the HLA-C locus was confirmed.<sup>19</sup>

From 1980 to 1987, advances in biochemistry, molecular biology, and the development of monoclonal antibodies to the MHC antigenic product markedly expanded knowledge about the MHC. Workshops during this time confirmed the existence of the DQ and DP loci<sup>20,21</sup> and focused on increasingly refined methods for identifying the burgeoning number of HLA alleles and correlation of the serologic, biochemical, and molecular biologic findings related to the MHC. In the 1996 and subsequent two workshops, a primary focus was the use of powerful genomic technology to identify the more than 2000 MHC alleles now known to exist in human populations around the world.

**Major Histocompatibility Complex**

The Class I, II, and III genes of the MHC lie in a 4-megabase region of chromosome 6 (8 megabases if one includes the more telomeric Class I-related gene HFE and the butyrophilin family of genes.)<sup>22</sup>

**Table 57-1** Historical Overview of International Histocompatibility Workshops

| International Workshops | Year | Major Theme                                                                                                                       |
|-------------------------|------|-----------------------------------------------------------------------------------------------------------------------------------|
| I                       | 1964 | Comparison of serologic techniques                                                                                                |
| II                      | 1965 | Standardized techniques and nomenclature                                                                                          |
| III                     | 1967 | One major genetic system named HL-A                                                                                               |
| IV                      | 1970 | International anti-sera analysis                                                                                                  |
| V                       | 1972 | Population differences for HLA                                                                                                    |
| VI                      | 1975 | Identification of D(DR) locus by cellular techniques; HLA-C locus confirmed                                                       |
| VII                     | 1977 | Serologic detection of DR antigens                                                                                                |
| VIII                    | 1980 | Compendium of HLA antigen frequencies                                                                                             |
| IX                      | 1984 | Introduction of RFLP molecular techniques; DQ/DP loci confirmed                                                                   |
| X                       | 1987 | Standardization and application of RFLP; introduction of PCR/SSOPH typing                                                         |
| XI                      | 1991 | Standardization and application of PCR/SSOPH HLA Class II typing to transplantation, disease association, and population genetics |
| XII                     | 1996 | Analysis of Class I and Class II alleles by means of DNA sequencing                                                               |
| XIII                    | 2002 | Definition of extent of Class I and II gene variation and application to typing of volunteer donors                               |
| XIV                     | 2005 | Genomics and immune responses                                                                                                     |

RFLP = restriction fragment length polymorphism; PCR = polymerase chain reaction; SSOPH = sequence-specific oligonucleotide probe hybridization.

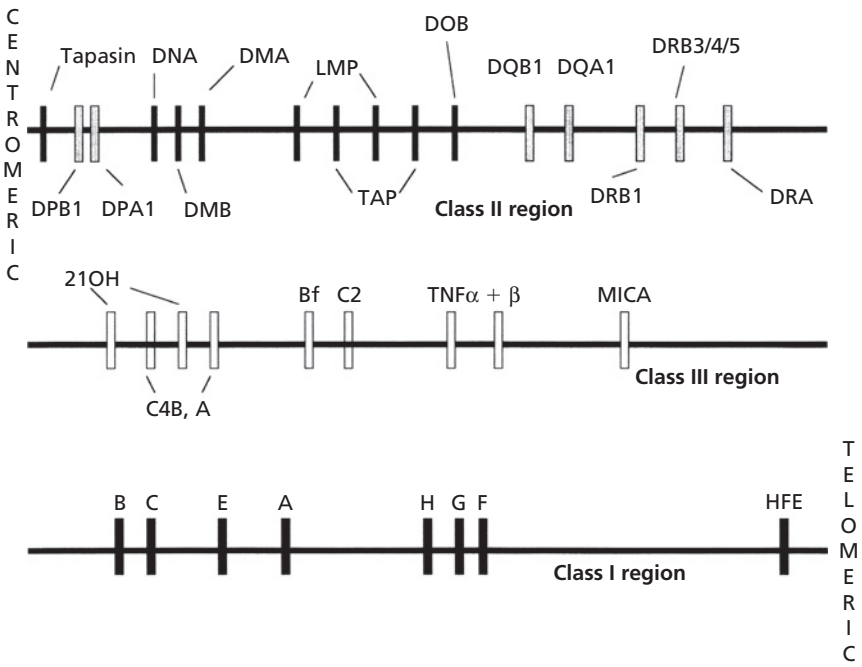


**Figure 57-1.** Selected genes in the major histocompatibility region on human chromosome 6 in the Class I, II, and III regions. Distances between loci are not drawn to scale. For exact gene locations, see <http://www.sanger.ac.uk/HGP/Chr6/MHC.shtml>.

The telomeric 2000kb of the MHC contains a number of genes, including those encoding the Class I antigens (Fig 57-1). The Class I HLA-A, -B, and -C genes reside in this region, as do the nonclassical MHC-Ib genes HLA-E, -F, and -G. HLA-E, -F, and -G are expressed at lower levels than are the classical genes, do not have the extensive polymorphism of the HLA-A, -B, and -C genes, and appear to have more limited functions in the immune system. Between the HLA-B locus and the tumor necrosis factor loci described later resides the MHC Class I chain-related

genes that encode variants of Class I proteins. Loci within this family display extensive polymorphism and encode proteins that interact with T cells and participate in natural killer cell activation.<sup>23</sup> Finally, the HFE gene responsible for genetic hemochromatosis lies approximately 4.3 megabases telomeric to HLA-A.<sup>24</sup> Centromeric of the HLA-B locus of the Class I region is a 1-megabase segment traditionally called the Class III region (Fig 57-1).<sup>22</sup> This region has a dense and diverse array of genes, many with as yet unknown functions. Some of these Class III

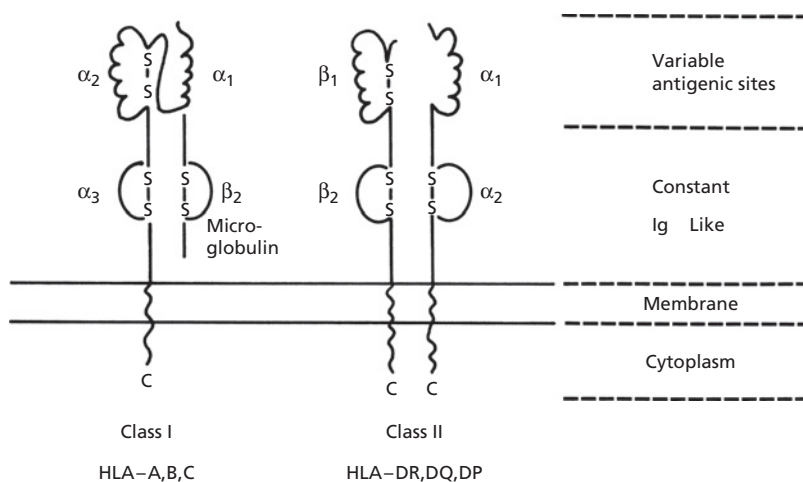


**Figure 57-2.** Simplified illustration shows the biochemical structure of Class I and II antigens. Modified with permission from Strominger.<sup>27</sup>

genes encode proteins with functions in innate immunity and inflammation. This region includes the complement components C2, Bf (factor B protein of the alternate pathway of complement activation), and C4. In most persons, the C4 locus is duplicated as C4A and C4B, each encoding a functional, somewhat biochemically different, molecule. Members of the tumor necrosis family also lie within the Class III region. Other genes within the Class III region, such as those encoding 21-hydroxylase enzymes involved in steroid metabolism, have no direct link to immunity. Although linkage to specific HLA alleles may be helpful in assessing 21-hydroxylase deficiency associated with congenital adrenal hyperplasia, direct genotyping is now possible.

The Class II gene loci occupy approximately 1 megabase of DNA centromeric to the Class III region (Fig 57-1).<sup>22</sup> Within this region are 18 closely linked loci that code for the  $\alpha$  and  $\beta$  chains of the Class II antigenic proteins. Proceeding from the telomeric to the centromeric boundaries of the region, the first gene cluster is composed of several  $\beta$ -chain loci and one  $\alpha$ -chain locus that encode the HLA-DR antigens. The DRB1 locus is responsible for the DR1 to DR18 specificities, whereas the DRB3, DRB4, and DRB5 loci are responsible for the DR52, DR53, and DR51 specificities, respectively. Each DR specificity is formed by a heterodimer encoded by the DRB1, 3, 4, or 5 locus and the DRA locus. Centromeric to the DRB genes are the DQ genes (Fig 57-1). The DQA1 and DQB1 loci encode the HLA-DQ specificities. Near the centromeric end of the MHC, the genes DPA1 and DPB1 encode the HLA-DPw1 to DPw6 specificities. The Class II region also contains several pseudogenes related to the genes encoding the DR, DQ, and DP antigens with errors preventing successful transcription and translation. Other genes with important accessory functions to Class I and II antigen presentation are present in the Class II region. The LMP2 and LMP7 genes, the TAP1 and TAP2 genes, and the tapasin gene encode proteins that participate in protein degradation and peptide transport and loading in the Class I system.<sup>25</sup> The HLA-DM and HLA-DO loci encode proteins involved in peptide loading in the Class II system.

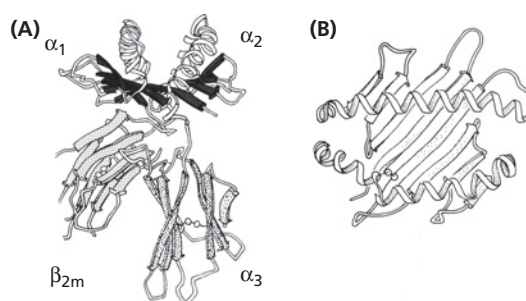


**Figure 57-3.** (A) Side view of Class I antigen molecule. (B) View of the antigen-binding pocket looking down toward the cell membrane. Modified with permission from Bjorkman et al.<sup>28</sup>

## Class I and II Antigens and Their Function

The structure, tissue distribution, and function of the glycoprotein antigenic products of the Class I and Class II genes of the MHC differ considerably. Both Class I and Class II antigens are heterodimeric, having three to four extracellular domains in addition to transmembrane and intracytoplasmic regions (Fig 57-2).<sup>26</sup> Class I antigens are composed of a 45,000-D  $\alpha$  heavy chain encoded by the HLA-A, -B, and -C gene loci non-covalently bound to a 12,000-D light chain,  $\beta_2$ -microglobulin, encoded by a gene locus on chromosome 12. The  $\alpha$  chain is a transmembrane protein, whereas  $\beta_2$ -microglobulin is extracellular. Domains  $\alpha_1$  and  $\alpha_2$  contain variable amino acid sequences and thus represent the antigenic sites. The  $\alpha_3$  domain has constant sequences homologous to the constant regions of immunoglobulin proteins. The  $\alpha_3$  domain of the heavy chain and  $\beta_2$ -microglobulin are noncovalently associated with each other near the cell membrane. The  $\alpha_1$  and  $\alpha_2$  domains sit on top of them to form peptide antigen-binding cleft with a floor of eight flat  $\beta$  sheets bound on the sides by two long  $\alpha$  helices, as illustrated in Fig 57-3. Most of the polymorphic amino acid changes

of the Class I histocompatibility antigen differences are associated with the floor or sides of this pocket, although some map to other domains.<sup>28,29</sup>

Class II antigens are composed of a 33,000-D  $\alpha$  chain non-covalently associated with a 28,000-D  $\beta$  chain (Fig 57-2). The  $\alpha$  chain is encoded by the A gene loci and the  $\beta$  chain by the B gene loci in the HLA-D region of the MHC. For example, the DQ antigen  $\alpha$  and  $\beta$  chains are encoded by the DQA1 and DQB1 genes, respectively. The  $\alpha_1$  and  $\beta_1$  domains are variable and form an antigen-binding groove similar to that described earlier.<sup>30</sup> The  $\alpha_2$  and  $\beta_2$  domains are relatively constant with immunoglobulin-like homology.

Class I antigens have a universal tissue distribution as plasma membrane proteins of all nucleated cells and platelets. Class II antigens have a limited tissue distribution primarily on antigen-presenting cells such as B lymphocytes and macrophages, although they can be induced in several cell types, including endothelial cells.

Class I and II molecules acquire self and nonself peptides in their binding cleft and display them on the plasma membrane of cells for recognition by the T-cell antigen receptor.<sup>25</sup> In the normal state, T cells that bind with high affinity to self peptides in the context of an individual's Class I and II molecules are either deleted or suppressed by a variety of mechanisms to prevent autoimmune disorders. However, peptides derived from viruses, bacteria, and some parasites presented by the Class I and Class II molecules to T-cell antigen receptors evoke an immune response. Class I molecules primarily acquire 8- to 10-amino-acid peptides generated by means of degradation of cytoplasmic proteins in the proteosome system. Class I antigens function as MHC restriction elements in the destruction of virus-infected target cells and present peptides to CD8 cytotoxic T cells. Class II molecules bind 13- to 25-amino-acid peptides of exogenous and endogenous origin degraded within the endosomal system. Class II antigens are MHC restriction elements that augment the sensitizing limb of the immune response and present peptides to CD4

helper T cells. Activation of CD8 and CD4 T cells then results in a program of cell division and differentiation resulting in cellular and humoral immune responses.<sup>25</sup>

Nomenclature and Polymorphism of the HLA System

Table 57-2 illustrates the HLA gene loci, the variable polypeptide chains, the number of related serologic specificities, and the number of alleles within each specificity. In the Class I region, the HLA-A, -B, and -C loci are all highly polymorphic with 300 to 1000 known alleles.<sup>31</sup> Multiple alleles are present within most of the known serotypes. For example, the A2 serotype has more than 160 alleles. In the Class II region, the HLA-DRA locus is almost monomorphic, whereas the DRB genes are polymorphic, with hundreds of known alleles. The DQA1 and DQB1 genes and the DPA1 and DPB1 loci demonstrate similar but less extensive polymorphism. The DQA1, DPA1, and DPB1 allelic variation can be detected with DNA typing but not with serologic testing.<sup>32</sup> The HLA system is the most polymorphic genetic system known to exist in humans. The number of different phenotypes possible from all combinations of these HLA alleles is greater than the global population. Fortunately, linkage disequilibrium in all human populations results in great overrepresentation of certain haplotypes, which makes finding HLA-identical individuals within a population for purposes such as unrelated marrow transplantation possible.

Exons in which most of the polymorphism in the Class I (exons 2 and 3) and Class II (exon 2) genes occurs encode the  $\alpha_1$  and  $\alpha_2$  and the  $\alpha_1$  and  $\beta_1$  domains, respectively, which interact with bound peptides.<sup>31</sup> New alleles appear to emerge at a fairly high rate and become fixed in populations, in theory, if they provide a selective advantage in presenting peptides from infectious organisms. New alleles are generated by means of point mutation, recombination, and gene conversion-like events.<sup>31,32</sup>

Table 57-2. HLA Nomenclature

| Genetic Locus | Encoded Polypeptide | Antigen or Associated Specificity | Allelic Equivalent | Number of Known Alleles/ Polypeptides |
|---------------|---------------------|-----------------------------------|--------------------|---------------------------------------|
| HLA-A         | $\alpha$            | A1 to A80                         | A*0101 to *8001    | 630/495                               |
| HLA-B         | $\alpha$            | B7 to B81                         | B*0702 to *8301    | 979/833                               |
| HLA-C         | $\alpha$            | Cw1 to Cw10                       | Cw*0102 to *1803   | 338/264                               |
| DRA           | $\alpha$            |                                   | DRA*0101 to *0102  | 3/2                                   |
| DRB1          | $\beta_1$           |                                   | DRB1*0101 to *1612 | 549/462                               |
| DQA1          | $\alpha$            | DQ1 to DQ9                        | DQA1*0101 to *0602 | 34/25                                 |
| DQB1          | $\beta_1$           |                                   | DQB1*0501 to *0634 | 90/66                                 |
| DPA1          | $\alpha_1$          | DPw1 to DPw6                      | DPA1*0103 to *0401 | 24/15                                 |
| DPB1          | $\beta_1$           |                                   | DPB1*0101 to *9901 | 128/114                               |

The number of polypeptides known for each locus reflects unique amino acid sequences encoded by a larger number of alleles that may differ only by silent polymorphisms.

The nomenclature of the HLA system is established by an international committee sponsored by the World Health Organization and is updated frequently.<sup>33,34</sup> Table 57-2 shows the general scheme for designating HLA antigens and alleles. Each serologic specificity is prefixed by the genetic system designation HLA-, followed by a letter denoting the encoded antigen (eg, A, B, C, DR). This letter is followed by a digit indicating specificity.<sup>33</sup> For example the A locus encodes the specificities HLA-A1 through A80; the B locus, HLA-B7 through B81; the C locus, HLA-Cw1 through Cw10; the DRA and DRB1 loci, HLA-DR1 through DR18; and the DQB1 and DQA1 loci, HLA-DQ1 through DQ9.

The nomenclature of the HLA system was first established with serologic data. Molecular data were introduced later according to the precise DNA sequences of each allele. The existing nomenclature had to be modified to accommodate this information. This modification is shown in Table 57-2. For Class I alleles, because only the  $\beta$  chain is variable, the molecular designation is the system (HLA-), the locus (A, B, or C), an asterisk (\*), and a four-digit number. The first two digits of this number correspond to the serologic specificity; for example, *HLA-A\*0301* is the first molecular allele of the HLA-A3 serologic specificity, and *HLA-A\*0302* is the second. In the Class II region, because both the  $\alpha$  and  $\beta$  chains can be variable, the locus designation must include the polypeptide chain responsible for the allele. For example, Class II, *HLA-DRB1\*1501* is interpreted as HLA system, DR locus,  $\beta$ -1 polypeptide chain (B1), DR15 serologic specificity (15 after the asterisk), first molecular allele (01). For molecular alleles that have no serologic equivalents, alleles are sequentially numbered; for example, *HLA-DQA1\*0101* is the first molecular allele of the  $\alpha_1$  polypeptide chain of the DQ. Fifth, sixth, and seventh digits are employed to designate alleles with silent polymorphisms or with variation occurring outside exons.

## Identification of HLA Antigens and Alleles

The detection of HLA antigens and alleles is generally accomplished with three basic methods (Table 57-3). The microlymphocytotoxicity assay developed by Terasaki and McClelland<sup>7</sup> of peripheral blood lymphocytes isolated from whole blood has been the standard test for the serologic detection of HLA-A, -B, and -C antigens. This is a complement-dependent test in which the addition of rabbit serum to HLA antibodies fixed to the lymphocyte membrane causes cell death. The endpoint is leakage or retention of fluorescent dyes from lymphocytes with damaged or intact cell membranes, respectively. Modifications of this test, primarily an increase in incubation time and isolation of B lymphocytes from peripheral blood, allow typing for the HLA-DR and -DQ antigens.<sup>17</sup>

Variations of the mixed leukocyte reaction (Table 57-3) with homozygous testing cells, primed lymphocytes, or T-cell clones are the primary methods for detecting HLA-D, -DQ, and -DP antigens.<sup>35-37</sup> The mixed leukocyte reaction methods have been

largely replaced by nucleic-acid-based techniques for the detection of Class II alleles.

The application of molecular genetic technology to the field of histocompatibility has led to an unprecedented expansion in knowledge of the MHC at the molecular level. DNA sequences have been obtained for the HLA genes and alleles in a variety of populations worldwide.<sup>33</sup> These sequence data have been useful for several reasons.

1. They have revealed the underlying DNA sequence variations among individuals (and therefore, amino acid variations) responsible for the antigenic differences in HLA molecules detectable with traditional serologic and cellular HLA testing.
2. They have made apparent the fact that results of serologic HLA tests define only broad groups of Class I and Class II alleles. Numerous subgroups of alleles distinguishable by sequence differences are present within these broad serologic groups. The result is a very large number of known alleles.<sup>38</sup>
3. Sequence data have allowed detailed definition of the genetic basis of the HLA-mediated disease associations described later.
4. Knowledge of the DNA sequences of HLA alleles has facilitated the substitution of nucleic-acid-based methods for serologic and cellular methods for clinical HLA typing.

Several methods have been developed to identify Class I and II alleles. These range from restriction fragment length polymorphism assays to direct DNA sequencing (Table 57-3). Most laboratories have replaced or complemented serologic typing with DNA-based typing methods for several reasons. Precise identification of alleles not possible by serologic means is clinically important in unrelated bone transplantation, as discussed later. Second, nucleic-acid-based typing can help resolve serologic typings compromised by cross-reactivity or complicated by clinical states resulting in pancytopenia or poor expression of HLA antigens on lymphocyte membranes. Third, even in the best circumstances, serologic typing may not be accurate, especially in ethnic groups with great diversity at loci such as HLA-B.<sup>39</sup>

**Table 57-3.** Detection of HLA Antigens and Alleles

### Serologic methods

- HLA-A, -B, and -C: microlymphocytotoxicity test
- HLA-DR and DO: modified microlymphocytotoxicity test, B-cell-enriched lymphocytes

### Cellular methods

- HLA-D(DR) and DQ: one-way mixed lymphocyte reaction (MLR) with
  - Homozygous testing cells
  - Primed lymphocytes (PLT)
  - Cloned T cells
- HLA-DP: MLR in PLT or with T-cell clones

### Nucleic-acid-based methods for Class I and II allele identification

- Sequence-specific primer polymerase chain reaction
- Sequence-specific oligonucleotide probe hybridization analysis
- Automated DNA sequencing
- Emerging DNA sequencing techniques

### Sequence-Specific Oligonucleotide Probe Hybridization

Relevant regions of the Class I and Class II genes are amplified from genomic DNA by means of the polymerase chain reaction (PCR) with two oligonucleotide primers that anneal to 5' and 3' flanking regions that are conserved (are identical) among individuals. Care must be taken in choosing primers to find ones that are locus-specific (amplify HLA-A but not the related HLA-B locus, for example), that amplify all known alleles at a locus, and that result in roughly equal amplification of the two alleles in a heterozygous individual. After the PCR, the amplified DNA is generally interrogated with allele-specific DNA probes attached to solid supports. Identification of the individual alleles is accomplished by means of hybridization of the PCR products with a series of fluorescently labeled sequence-specific oligonucleotide probes.<sup>40,41</sup> Probes are chosen to anneal to amplified regions of the Class I and II genes that vary from allele to allele. Probes with nucleotide sequences perfectly complementary to the amplified DNA hybridize specifically to the PCR product target and can be detected via fluorescence. With careful control of stringency conditions of hybridization and washing, these probes can detect single-base-pair differences, which result in single-amino-acid substitutions in the expressed proteins. Alleles are assigned on the basis of panels of positive and negative hybridization reactions with oligonucleotides specific for a particular allele or sequence. Sequence-specific oligonucleotide probe hybridization (SSOPH) typing is complex because it requires a substantial number of oligonucleotide probes to detect and differentiate among the large number of known Class I and II alleles. However, the development of multiplexed methods for SSOPH employing fluorescent microspheres with attached allele-specific probes has greatly improved the ease of use and efficiency of this method and made it a leading means for HLA typing in many laboratories.<sup>42</sup>

### Sequence-Specific Primer Polymerase Chain Reaction

Polymerase chain reaction primers are designed so that their 3'-most one or two nucleotides are complementary to base positions within Class I and II genes that differ for different alleles.<sup>43</sup> Sequence-specific primers with 3' ends that anneal perfectly to sequences present in a particular allele or allele subgroup result in productive DNA amplification. Other alleles or allele subgroups are not amplified. Gel electrophoresis can then be used to detect the presence or absence of PCR products of the appropriate size, and hybridization assays are avoided. Sequence-specific PCR is most useful for medium-resolution HLA typing and for initial quick identification of broad antigen groups, such as DR52. Then, SSOPH or DNA sequencing can be used to define microvariants of these specificities, if necessary. Sequence-specific PCR requires 100 or more simultaneously performed PCR assays per patient to identify HLA-A, -B, and -DRB allele groups at serologically equivalent resolution. The method requires optimization to avoid failed amplification reactions in each run.

### Sequence-Based Typing

Sequence-based typing has the advantage of requiring only a limited number of primers for the PCR for each HLA locus.

There is no need for a series of detecting oligonucleotide probes for each allele. Polymerase chain reaction products are prepared that will encompass the relevant polymorphic positions within the Class I or II gene to be sequenced. Sequencing primers are annealed to the PCR products to generate sequencing ladders that are separated on a high-resolution gel. Any known or previously unknown allele in the population can be identified by means of inspection of the DNA sequence or electropherogram. Electropherograms must be of high quality for accurate detection of heterozygous positions in the sequenced PCR products. The development of high-throughput automated fluorescent DNA-sequencing machines, analysis software, and capillary electrophoresis systems has made this method less cumbersome. Direct sequencing for HLA typing yields unparalleled precision in allele identification. As the number of known Class I and II alleles continues to escalate, the SSOPH and sequence-specific PCR methods have become increasingly complex, making direct sequencing an increasingly attractive method for laboratories.<sup>44-46</sup>

### Choice of HLA Typing Method

Several factors influence a decision to use one HLA typing method over another. Sequence-specific PCR and reverse SSOPH can be performed in a time (4 to 5 hours) appropriate for clinical situations such as cadaveric renal transplantation. DNA sequencing is a reasonable approach for situations such as unrelated marrow transplantation in which allele-level HLA matching of donors and recipients is desired and turnaround times of a few days to a week are acceptable.

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## Genotypes, Phenotypes, and Haplotypes

HLA antigens are expressed codominantly. The phenotype of expressed antigens for any given person is not equivalent to the genotype. Because HLA genes are closely linked on chromosome 6, they are inherited in units known as haplotypes. Each sibling of a family inherits one of two HLA haplotypes from each parent.

Figure 57-4 illustrates the segregation of haplotypes in a family of seven, focusing on the A, B, Cw, and DR serologic specificities. Analysis of the data for a single individual does not allow assignment of the serologic types present to a specific paternally or maternally derived chromosome; that is, the typing data are unphased. Thus, one cannot be certain whether the DR7 in sibling 3 is present on a chromosome carrying B8 or B13. However, analysis of the typing data for the entire family and the transmission of specificities to the children make it possible to phase each of the pairs of parental chromosomes and to determine haplotypes. For example, one of the paternal chromosome-6 pairs, haplotype [a], contains HLA-A1, Cw4, B35, and DR1; the other paternal chromosome, haplotype [b], includes HLA-A3, Cw7, B8, and DR7. Similarly, the maternal chromosome-6 pairs can be separated as haplotypes [c] and [d]. Each child in this family inherits [a] or [b] from the father and [c] or [d] from

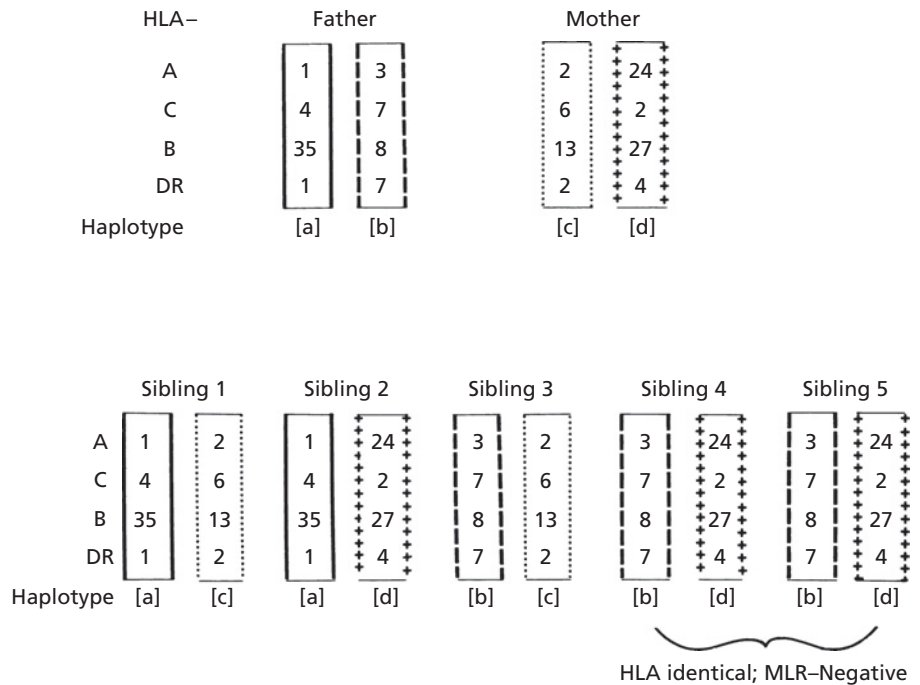


**Figure 57-4.** HLA-A, -B, -C, and -DR antigens segregating as haplotypes in a family. The data reflect the genotypes of each family member as well as the parental haplotypes.

the mother. Each child differs from each parent by one HLA haplotype. Because there can be only four parental haplotypes, the chances are 1 in 4 that the siblings will have same paternal-maternal haplotypes (are HLA-identical), 1 in 2 that siblings will differ by one haplotype, and 1 in 4 that they will differ by two haplotypes. For example, siblings 4 and 5 are HLA-identical, and sibling 4 shares one haplotype with siblings 2 and 3 but no haplotypes with sibling 1. Because siblings 4 and 5 are genetically identical with regard to the MHC, their lymphocytes will not react in mixed leukocyte culture.

The difference between phenotypes and genotypes can be clarified even further with sibling 4 (Fig 57-4) as an example. If family data were not available, this sibling's phenotype would be written as HLA-A3, A24; Cw2, Cw7; B8, B27; DR4, DR7. This implies lack of family data that would allow assignment of the A, C, B, and DR combinations to the appropriate haplotypes. With family data available, however, the genotype is written HLA-A3, Cw7, B8, DR7/A24, Cw2, B27, DR4. The slash in this statement clearly defines the two haplotypes inherited by this child.

Although HLA gene loci are closely linked, there is a low frequency of recombination during meiosis. The crossover rate is approximately 0.8% between the A and B loci and approximately 0.5% between the B and DR loci. For example, 1 of every 200 families exhibits recombination between the B and DR genes.

HLA allele and haplotype frequencies exhibit ethnic variation. Some alleles and haplotypes are widely distributed around the globe, and others are almost exclusively within a particular ethnic group. In population studies, the phenomenon of linkage disequilibrium is apparent in all groups. This term indicates that in randomly mating populations, the haplotype frequency for

two or more linked gene loci is significantly higher than would be expected by chance alone. The expected frequency is obtained from the product of the gene frequencies of the involved genes. For example, among persons of European ancestry, the observed A1, B8 haplotype frequency (7.0%) is approximately 4.4 times greater than the expected haplotype frequency (1.6%). These excess haplotype frequencies may exist for a variety of reasons, including high prevalence in the founders of a population and selective pressure from infectious organisms.<sup>47</sup>

## Medical and Biologic Significance of HLA

The HLA system plays a role in several areas of biomedical significance including the following:

- Antigen presentation
- Association with certain diseases
- Organ and stem cell transplantation
- Platelet transfusion
- Population genetics and anthropology.

Tracing the appearance of novel HLA alleles in populations and comparing the frequencies of alleles among populations to gain information about human origins and migration in anthropologic and evolutionary studies is a fascinating field of inquiry but beyond the scope of this chapter.<sup>48</sup> The extensive polymorphism of the HLA loci has been exploited for many years in identity testing; however, the use of microsatellite loci for this purpose has supplanted the use of HLA loci. The role of HLA Class I typing to support platelet transfusion is described in Chapter 11.



## Transplantation

Transplantation poses a special problem in medicine because the extreme polymorphism of the HLA loci make it unlikely that an unrelated donor of cells, tissues, or organs will be matched at an allele level or at a serologic level with a recipient without a concerted effort to identify matched donors and recipients. One solution is to identify an HLA-identical sibling, but many potential transplant recipients do not have access to these donors. For organ transplantation, powerful immunosuppressant drugs have made transplantation feasible with mismatched living and cadaveric donors and recipients. However, better HLA-A, -B, and -DR matching at a serologic level generally increases the half-life of the transplanted organ and decreases overall morbidity.<sup>49</sup> Thus cadaveric donor kidneys are shared on a national basis with waiting recipients who have end-stage renal failure and no mismatches.<sup>50</sup>

Finding well-matched donors of hematopoietic stem cells if there are no HLA-identical siblings is facilitated by resources such as the National Marrow Donor Program, which has enrolled several million potential donors with known HLA types.<sup>51</sup> The goal for unrelated stem cell donation is increasingly allele-level matches for HLA-A, -B, -C, and -DRB1, although the level of mismatching permissible is incompletely understood. Subtle allelic mismatches between stem cell donors and recipients appear to increase the risk of severe graft-vs-host disease and decrease overall survival.<sup>52,53</sup> The use of umbilical cord blood stem cells and the depletion of T cells from transplanted cells may allow greater donor-recipient mismatches.<sup>54,55</sup> Retrospective studies are continuing to assess in a definitive manner the level of matching necessary for successful stem cell transplantation.<sup>56</sup> Hematopoietic stem cell transplantation is discussed in Chapters 33 through 36.

There are two major mechanisms by which Class I and II donor-recipient mismatches may adversely affect transplantation—production of HLA antibodies and cell-mediated rejection. Transplant recipients become sensitized or produce antibodies directed against Class I and Class II specificities through several routes. Exposure to fetal HLA molecules encoded by paternal haplotypes during pregnancy, especially multiple pregnancies, can lead to the presence of long-lasting, high-titer HLA antibodies. Similarly, mismatched HLA antigens from previous organ donors and donors of transfused blood components can lead to sensitization. The presence of recipient preformed HLA antibodies may lead to hyperacute rejection of a donor organ bearing the relevant HLA specificities.<sup>26</sup> Therefore, laboratories maintain serum screening programs to detect and identify HLA antibodies in waiting recipients so that inappropriate donors can be avoided. Immediately before transplantation, crossmatches to detect donor-directed HLA antibodies are performed between donor lymphocytes and recipient serum by means of the microcytotoxicity assays described earlier or more sensitive flow cytometric technology.<sup>57</sup>

Donor HLA antigens mismatched with recipient HLA antigens can elicit a strong cell-mediated immune response. As many as 10% of a recipient's T cells are able to recognize and respond to donor-mismatched HLA antigen.<sup>58</sup> These responses

contribute to acute rejection in organ transplantation and graft-vs-host disease in stem cell transplantation. HLA matching and immunosuppressive therapy help to mitigate this problem.

## Disease Association

The association of certain HLA alleles and their encoded antigens with particular diseases, especially autoimmune disorders, has been known for some time. In more than 40 disorders, there is significant deviation in the frequency of HLA antigens from that of healthy controls (Table 57-4).<sup>59</sup> In general, HLA-associated diseases have certain common features. They are known or suspected to have an inherited component, usually have autoimmune features, and display a clinical course often featuring repeated acute relapses followed by remission. For most HLA-associated diseases, the etiologic factor is unknown and the pathophysiologic mechanism is incompletely understood. One hypothesis for the link between HLA antigens and disease suggests that a necessary but not sufficient requirement for disease development is the differential ability of a Class I or II heterodimer encoded by a specific allele to present autoantigens to the T-cell receptor.

Two of the strongest HLA associations are the *DQB1\*0602* allele with narcolepsy<sup>60</sup> and *B\*27* alleles with ankylosing spondylitis.<sup>59</sup> Almost all patients with narcolepsy have the associated alleles; approximately 90% of patients with ankylosing spondylitis have the associated alleles. In populations without these disorders, the frequencies of *DQB1\*0602* (25% to 30%) and of the *B\*27* group (5% to 10%) are substantial but do not approach the frequencies among persons with these disorders. Thus the antigens involved are not unique to narcolepsy and ankylosing spondylitis but are overrepresented among affected persons. Approximately 3% of persons with a *B\*27* allele have ankylosing spondylitis, a risk approximately 100-fold greater than that among persons without *B\*27*. Results of HLA testing that show the absence of *DQB1\*0602* or *B\*27* alleles is useful to help rule out a diagnosis of narcolepsy or ankylosing spondylitis for a patient. Results that show the presence of these alleles are less useful because of the prevalence of these alleles in the general population.

The association of HLA antigens with most other autoimmune disorders usually does not carry the high relative risk that narcolepsy and ankylosing spondylitis do. Although these HLA associations are generally less useful in diagnosis, they provide important insights into the pathophysiologic mechanism of these diseases and may help assess prognosis. For example, approximately 95% of patients with type 1 diabetes mellitus (formerly known as insulin-dependent diabetes mellitus) have either the *DRB1\*03* or the *DRB1\*04* allele or both.<sup>61</sup> Patients heterozygous for *DRB1\*03\*04* have a relative risk for type 1 diabetes mellitus three to six times greater than that of patients with *DRB1\*03* or *DRB1\*04* alone or in combination with another *DRB1* allele. Susceptibility to type 1 diabetes mellitus appears to be linked to haplotypes containing *DRB1\*03* or *DRB1\*04* with *DQB1\*02* and *DQB1\*03* alleles.

Many persons with rheumatoid arthritis have inherited *DRB1\*04* alleles encoding a common epitope. Heterozygosity

**Table 57-4.** Selected Diseases Associated with HLA Alleles

| Disorder                                                   | HLA Linkage                                                    | Comments                                                                                                             |
|------------------------------------------------------------|----------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------|
| <b>Class I and II Associations</b>                         |                                                                |                                                                                                                      |
| Ankylosing spondylitis                                     | <i>B*27</i> alleles                                            | Absence of <i>B*27</i> may be useful in ruling out diagnosis of ankylosing spondylitis                               |
| Narcolepsy                                                 | <i>DQB1*0602</i>                                               | <i>DQB1</i> locus or linked loci and alterations in hypocretin or orexin peptides/receptors contribute to narcolepsy |
| Type 1 diabetes mellitus                                   | <i>DRB1*03/*04</i> , <i>DQB1*02</i> , and <i>*03</i> alleles   | Presence of <i>DRB1*03/*04</i> confers a 25-fold relative risk                                                       |
| Rheumatoid arthritis                                       | <i>DRB1*04</i> alleles sharing epitope encoded by codons 67-74 | May be most useful in predicting severity of disease                                                                 |
| Latency period before onset of AIDS                        | Multiple loci and alleles                                      | Latency period shortened by homozygosity at loci and modified by multiple susceptibility and resistance alleles      |
| Abacavir hypersensitivity                                  | <i>B*5701</i>                                                  | Abacavir hypersensitivity largely a result of inheritance of <i>B*5701</i>                                           |
| <b>Mutation of Nonimmune Response Genes within the MHC</b> |                                                                |                                                                                                                      |
| 21-Hydroxylase deficiency                                  | Diagnosis by direct <i>CYP21</i> genotyping                    | Linked to cosegregating HLA haplotypes in families                                                                   |
| Complement component 2 (C2) deficiency                     | Diagnosis by assessment of complement levels                   |                                                                                                                      |
| Hemochromatosis                                            | Diagnosis by direct <i>HFE</i> genotyping                      | <i>HFE</i> is a Class-I-related gene at the telomeric end of the MHC                                                 |

and homozygosity for alleles encoding this epitope may be predictive of a more severe course of arthritis. Thus allele identification may be helpful in assessing prognosis.<sup>62</sup>

A number of studies have been performed in an attempt to define specific HLA alleles that confer susceptibility or resistance to AIDS (Table 57-4).<sup>63,64</sup> There appear to be HLA alleles that interact with other genetic and environmental factors to influence the outcome of this multifactorial disease. However, results may vary somewhat among studies given the complexity of AIDS, differences in the ethnicity of the populations studied, and the subtypes of the human immunodeficiency virus type 1 involved. Recently, hypersensitivity to the antiretroviral drug abacavir was linked to the presence of *HLA-B\*5701* in treated patients.<sup>65,66</sup> Near complete elimination of hypersensitivity morbidity was achieved by first screening patients to exclude the 5% to 10% with *B\*5701* from exposure to this agent. In this example, HLA allele identification serves as a pharmacogenomic test to stratify a patient population for targeted therapy.

Because the MHC contains genes with no obvious role in immune system function, some diseases caused by point mutation or deletion of genes in this region cause disorders not directly related to immunity. However, because of linkage disequilibrium, overrepresentation of specific Class I or Class II antigens may occur in these disorders. Congenital adrenal hyperplasia is caused by mutations in the MHC Class III region genes encoding 21-hydroxylase.<sup>67</sup> Although linkage to specific HLA alleles is present in persons with congenital adrenal hyperplasia, direct genotyping is generally preferable for diagnostic purposes. Similarly, genetic hemochromatosis is caused by mutations in the *HFE* gene telomeric to the *HLA-A* locus.<sup>24</sup> Although the presence of *A\*03* confers several-fold excess risk of hemochromatosis, the most direct route to genetic diagnosis is *HFE* genotyping.

## Summary

The HLA loci on chromosome 6 are composed of a series of Class I and II genes that display a degree of naturally occurring polymorphism unmatched within the rest of the human genome. This polymorphism appears to be maintained to ensure appropriate responses to the infectious organisms encountered by global human populations. The crucial role of the Class I and II molecules is to bind to the degradation products of proteins and present them to T-cell antigen receptors for recognition as endogenous or foreign peptides. MHC polymorphism has several consequences in medicine and biology beyond the normal immune response: 1) MHC genetic variation can be exploited as a tool for anthropologic study of human migration and development. 2) Specific HLA alleles are associated with a propensity to development of particular disease entities, especially autoimmune disorders. 3) The challenge of finding HLA-matched donors and recipients presents special problems in stem cell and organ transplantation.

## Disclaimer

The author has disclosed no conflicts of interest.

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In the United States, organ and tissue procurement has been organized in such a way so as to maximize availability in an efficient fashion that is fair to both donor and recipient. Most of the organ procurement efforts have become systematized and organized under federal mandate; there is, however, a growing cooperation between organ procurement organizations (OPOs) and tissue recovery organizations to facilitate meeting the requirements of both organ and tissue procurement. Successful tissue preservation has encouraged the use of bone and soft tissue grafts, cardiovascular tissue, and skin in the clinical setting. Semen cryobanking has been practiced for many years, and the demand for embryo banking services has greatly increased recently as assisted reproductive procedures have become more diverse and much more widely available. As technology in this area continues to expand, blood banks may play an increasingly important role in coordinating, supporting, and directing transplantation and transplant-related interventions.

### Growth of Tissue Banking

Each year, hundreds of thousands of patients benefit from donated bone, cartilage, and tendon used to reconstruct and rehabilitate joints and other bony structures, corneas to restore sight, skin to treat burns, veins and great vessels to restore blood flow, heart valves to restore cardiac function, and reproductive tissue to treat infertility. In addition to whole grafts and sections, bone, in particular, can be machined into specialized products to conform with manufactured support constructs. It can also be ground into demineralized bone matrix (DBM), which is available as a ground powder or as combination forms such as gels, pastes, strips, and even moldable grafts designed to avoid migration in specific applications. Myriad types of tissues are transplanted in a variety of medical and dental specialties for

diverse clinical applications (Table 58-1).<sup>1</sup> The most commonly transplanted allografts are bone and cornea (Table 58-2).

The total number and range of tissues collected, processed, and transplanted in the United States are difficult to determine, because there is no national reporting system. In 1999, tissue banks accredited by the American Association of Tissue Banks (AATB) distributed approximately 750,000 allografts for transplantation. By 2003, the figure had more than doubled. The American Orthopaedic Society for Sports Medicine reported that its members used more than 60,000 allografts in knee surgery alone, in 2005.<sup>2</sup> The quantity of musculoskeletal tissue allografts used annually is approximately 1,500,000. Cornea transplants numbered >45,000 in 2006.<sup>2</sup> Skin is distributed at the rate of about 17,000 square feet annually. Transplantation of heart valves is now estimated at >2500 annually. These procedures require annual donation of cardiovascular tissue, musculoskeletal tissue, and skin from >40,000 deceased donors (New York State Department of Health, unpublished data, 2005), and donation of eyes from >38,500 deceased donors.<sup>3</sup>

In addition to such tissues from deceased donors, many tissues for clinical use are derived from living donors, including semen and oocytes for use in artificial insemination and assisted reproductive technology procedures. In 2005, New York State-licensed semen banks located throughout the United States processed 27,118 ejaculates from 962 semen donors. In addition, semen from 16,347 client depositors was collected and cryopreserved for later use by the client depositor's wife or other "intimate partner" (New York State Department of Health, unpublished data, 2005). Donor oocytes were used in approximately 12% of all assisted reproductive technology cycles carried out in the United States in 2005.<sup>4</sup> Tissue from living donors is also considered by some to include human milk to nourish low-birthweight, premature infants. The Human Milk Banking Association of North America reports the existence of 11 active member banks.<sup>5</sup> The Food and Drug Administration (FDA), however, does not consider human milk to be subject to regulations that apply to human allograft tissue.

Because no comprehensive national usage figures are available, it is difficult to determine the rate of increase in the demand for

**Table 58-1.** Some Common Human Allograft Applications, by Specialty<sup>1</sup>

| Specialty                  | Procedure/Application                                                                                                                                                                                                                                                   | Allograft Types Used                                                                                                                                                                                                                                                                                                               |
|----------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| General orthopedics        | Trauma/fracture repair; osseous defect repair; acetabular repair; total joint revision/arthroplasty                                                                                                                                                                     | Femoral head; femoral condyle; whole, proximal, or distal bone shaft (femur, tibia, humerus); hemi-pelvis; cancellous bone; corticocancellous bone; cortical strut/screw/pin; bi-cortical strip; tri-cortical wedge; whole joint* (knee, ankle, shoulder, elbow); osteoarticular graft*; osteochondral graft*; DBM; osteobiologics |
| Sports medicine            | Tendon, anterior cruciate ligament, posterior cruciate ligament, other knee ligament repair; meniscus repair/replacement; osteochondral defects; rotator cuff repair; ankle/tendon ligament repair                                                                      | Patellar ligament; Achilles tendon; tibialis tendon; semitendinosus tendon; gracilis tendon; peroneous longus tendon; fascia lata; rotator cuff; meniscus; meniscus w/tibial plateau; osteochondral plug*; femoral hemi-condyle*; acellular dermal matrix                                                                          |
| Craniofacial/maxillofacial | Cranial reconstruction; maxillary/mandibular reconstruction; facial palsy repair                                                                                                                                                                                        | Mandible; dura mater*; pericardium; fascia lata; acellular dermal matrix; bi-cortical strip; tri-cortical wedge; DBM; osteobiologics; sclera                                                                                                                                                                                       |
| Dental                     | Alveolar ridge augmentation for dental implant placement; onlay grafting; sinus elevations/augmentation; socket/ridge preservation; intrabony defect repair                                                                                                             | DBM; osteobiologics; sclera; acellular dermal matrix                                                                                                                                                                                                                                                                               |
| Ophthalmology              | Postcataract corneal edema repair; Fuchs dystrophy repair; glaucoma drainage valve implantation; corneo-scleral fistula repair; keratoconus correction; phaco burn repair; orbital reconstruction following enucleation; eyelid ectropion repair; eyelid reconstruction | Cornea*; sclera*; pericardium                                                                                                                                                                                                                                                                                                      |
| Neurosurgical              | Cervical/lumbar interbody fusion; intermedullary rod placement; dura replacement                                                                                                                                                                                        | Dura mater*; fascia lata; pericardium; cancellous bone; corticocancellous bone; cortical strut; bi-cortical strip; various machined and constructed proprietary bone forms*; amniotic membrane*; acellular dermal matrix                                                                                                           |
| Burn treatment             | Wound covering <sup>†</sup>                                                                                                                                                                                                                                             | Fresh skin*; cryopreserved skin*; freeze-dried skin*; acellular dermal matrix; amniotic membrane*                                                                                                                                                                                                                                  |
| General surgery            | Urologic incontinence procedure; pelvic floor reconstruction; herniorrhaphy                                                                                                                                                                                             | Fascia lata; pericardium; lyophilized skin*; acellular dermal matrix                                                                                                                                                                                                                                                               |
| Cardiac                    | Congenital anomaly repair (both valve and outflow tract major vessel repair/replacement <sup>†</sup> ); cardiac valve and vessel repair/replacement; major vessel blood “shunting” procedures                                                                           | Aortic valve*; pulmonary valve*; various conduit-use-only grafts* from the ascending aorta or thoracic aorta or from the pulmonary artery trunk or its branches                                                                                                                                                                    |
| Vascular                   | Vaso-occlusive disease (peripheral, abdominal, thoracic); cardiac artery bypass grafting; arteriovenous shunt insertion; muscle-flap or organ transplant vascular bed extensions; replacement of infected prosthetic devices                                            | Greater saphenous vein*; aorto-iliac artery*; iliac vein*; iliac artery*                                                                                                                                                                                                                                                           |

\* Cannot be sterilized.

<sup>†</sup> Can be life saving.

DBM = demineralized bone matrix.

tissues. However, novel uses for tissue in transplantation have emerged that had not been envisioned even a few years ago. Amniotic membrane is transplanted to correct epithelial eye defects; bioengineered skin products are used for wound healing; and nerve tissue is transplanted to serve as a conduit for nerve regeneration in damaged limbs. In late 1998, the medical world was fascinated by the first hand transplant surgery; there have been at least 18 worldwide since, and the world's first face transplant procedure was performed in November 2005. In the future, human pluripotent progenitor cells from embryos may be used in the treatment of Parkinson disease, diabetes, and spinal cord injuries.

## Tissue Donation

### Living Donors

The tissue most commonly donated by living donors is blood, the primary subject of this book, but living donors also provide other tissues. Tissue donation by a living person generally is limited to renewable tissue, such as gametes, extraembryonic tissue, and milk. Except for autografts, which can be expanded by culturing for use on burned patients, skin is usually recovered from deceased donors. However, some tissue banks have

**Table 58-2.** Allogeneic Tissue Distributed for Clinical Use Annually in the United States

| Tissue                       | Units (Estimated) |
|------------------------------|-------------------|
| <b>Deceased Donor Tissue</b> |                   |
| Musculoskeletal tissue*      | 1,500,000         |
| Cornea†                      | 47,067            |
| Pericardium*                 | 11,304            |
| Tendon*                      | 32,441            |
| Skin (sq ft)*                | 16,597            |
| Fascia*                      | 16,754            |
| Heart valves‡                | 2,750             |
| Vessels‡                     | 3,000             |
| <b>Living Donor Tissue**</b> |                   |
| Semen (vials)‡               | >100,000          |
| Oocyte/embryos§              | 35,185            |
| Amniotic membrane‡           | 2,198             |

\*American Association of Tissue Banks. Annual survey of accredited tissue banks. McLean, VA: American Association of Tissue Banks, 2003.

†Eye Bank Association of America. 2006 Eye banking statistical report. Washington, DC: Eye Bank Association of America, 2007.

‡New York State Department of Health (NYSDOH). Unpublished projected data based on 74,366 semen vials/straws distributed by New York State-licensed tissue banks in 2005. (NYSDOH licenses semen banks located nationwide.)

§Centers for Disease Control and Prevention; American Society for Reproductive Medicine; Society for Assisted Reproductive Technology. 2005 Assisted reproductive technology success rates: National summary and fertility clinic reports. Atlanta, GA: Centers for Disease Control and Prevention; 2007.

developed processes to promote living donor skin donation following extensive weight loss (>300 such donations per year in the United States). Cartilage can also be cultured for autologous transplant in knee repair. Bone can be obtained from living donors in the form of femoral heads and tibial plateaus that are removed and would otherwise be discarded during surgical procedures (total hip and knee replacements with prostheses). Outside the United States, such surgical bone banking remains a significant source of bone grafts in many countries.

**Deceased Donors**

In contrast to organ donors, tissue donors (excluding living donors, of course) do not need to have functioning circulation. Tissues such as bone, eyes, and skin can generally be collected up to 24 hours after cessation of the donor’s cardiac and respiratory functions, depending on the temperature and environment in which the donor body is stored. Tissues such as bone and skin can be donated by deceased organ donors (a standard criterion for organ donors in the United States is brain death), but more commonly, tissues are donated by other hospitalized patients who have been judged deceased by both cardiorespiratory and neurologic criteria. Recently, tissue procurement practices have become more effective as a result of increased cooperation between tissue banks and OPOs, through routine referral requirements<sup>6</sup> and statutes (eg, New York State Public Health

Law §4351). Referrals for eye banks are also made through OPOs. However, many tissue banks, especially eye banks, obtain a significant portion of their referrals from medical examiners or coroners who have access to large numbers of young, healthy individuals who died suddenly and never reached a hospital.<sup>3</sup>

**Routine Referral for Donation**

The availability of tissues for transplantation depends on strong public support, the presence of laws that clear legal barriers to donation and that authorize consent before death (through such means as driver’s licenses, donor registries, and advance directives), and the availability of health professionals trained in how to approach the next of kin on the subject of donation. (The next of kin is/are a deceased person’s closest relative(s), as determined by a hierarchy specified in state law.) Key to donation in the United States is a spirit of altruism and volunteerism, coupled with autonomous choice being valued. Consent to donate is informed and voluntary with few exceptions. About one-third of eye banks are located in states with medical examiner laws that allow removal of corneas for transplantation under the following circumstances: 1) the body is under the medical examiner’s jurisdiction; 2) autopsy is required by law; 3) there is no known objection by the next of kin; and 4) the removal will not disturb the body’s appearance.<sup>3,7,8</sup> These state laws presume consent and permit removal of eyes without an interview of the next of kin concerning the deceased’s medical and social history, including human immunodeficiency virus (HIV) and hepatitis risk behaviors. Maryland expanded the law to cover non-medical-examiner cases when a next of kin cannot be found.<sup>9</sup> In 2006, >34,000 corneas were provided for transplantation; about 10% of these were obtained through use of a state’s medical examiner law. Nationwide the number of corneas obtained under medical examiner laws has been declining, mainly because of ethical concerns, but also because of increased FDA regulation that requires specific screening for relevant communicable diseases, which must include a donor medical and social history interview with a knowledgeable historian.<sup>3,7</sup>

In the late 1980s, most states enacted “required request” laws that sought to increase the supply of organs and tissues by requiring that hospital personnel request permission of the next of kin for organ and tissue donation at the time of a patient’s death (if the prospective donor was medically eligible). Following findings that families were more likely to consent to donation if approached by requesters who had received training in the most effective ways to present donation opportunities, some states amended these laws to require referral of all deaths to an OPO or designated tissue bank, whose specially trained requesters would then ask for consent. Federal routine referral requirements were implemented by the Health Care Financing Administration (now the Centers for Medicare and Medicaid Services) in Hospital Conditions of Participation for Organ, Tissues, and Eye Donation, which became effective August 21, 1998.<sup>6</sup> These rules mandate, as a condition of Medicaid and Medicare reimbursement, that all hospitals establish formal relationships with an

OPO in the service area, with a tissue bank, and with an eye bank. Further, the rules require that hospital personnel notify OPOs of all deaths and imminent deaths so that potential donors are identified, and so that the next of kin will be approached about donation, when appropriate. The effect of these routine referral laws is not clear. In New York, where such a law went into effect on January 1, 1998, mixed results have been reported. Although a small increase in tissue donation has been realized statewide, certain metropolitan areas have actually reported a decline in eye donation. It is also not clear whether the increase in tissue donation in recent years is the result of required referral or of enhanced advertising campaigns and increased public awareness of the need for organ and tissue donation. Under routine referral, the costs associated with around-the-clock reporting of deaths and imminent deaths are proving to be high in certain areas, although the cost to OPOs is shared by Medicare and the transplanting hospitals through “standard acquisition charges” applied to individual organs transplanted. Uniform Anatomical Gift Laws were developed to standardize state requirements for first party consent, requesting personnel, and recovery. A 2006 revised version, which expanded definitions, clarified roles, and enhanced a focus on personal autonomy, had been enacted in 20 states and had been introduced in 15 more by early 2008 (see <http://www.anatomicalgiftact.org>).

## Organization of Tissue Banking in the United States

Unlike the organ procurement and sharing system, tissue banking is not formally organized based on geography. There are approximately 15 independent tissue banks that process conventional musculoskeletal tissue, four that process cardiovascular tissue, and 13 that process skin. Although most are not for profit, a few for-profit companies process human tissue for transplantation. These processors usually independently perform donor eligibility assessment, including laboratory testing, even if the tissue bank that recovered the tissue had already performed such an assessment. An unpublished AATB survey of 2003 activity found that donor eligibility had been determined by a tissue bank accredited by AATB for about 95% of the bone grafts distributed (Scott Brubaker, personal communication, August 17, 2007). There are approximately 89 not-for-profit eye banks accredited by the Eye Bank Association of America (EBAA; see <http://www.restore-sight.org>). Almost all cornea allografts are provided by these eye banks. Blood centers with expertise in donor recruitment, donor eligibility determination, cell cryopreservation, and compliance with standards and regulations are good candidates to undertake tissue banking services. Some blood centers have already taken on the challenge of providing comprehensive tissue bank services, and they recover, process, store, and distribute tissues.

Procedures to achieve allograft safety and efficacy are guided by national professional standards set by such organizations as AATB,<sup>10</sup> EBAA,<sup>11</sup> and the American Society for Reproductive

Medicine.<sup>12</sup> The FDA and some states, including New York, Florida, and California, have regulatory and licensure requirements (see section on oversight of tissue banks below).

## Public Attitudes

Public altruism and the willingness of people to be organ and tissue donors form the foundation of the transplant system. A 1986 Gallup survey assessing the public’s attitudes found that people were more willing to consent to donation if they were provided with relevant information. Effective public education is thus a key component of successful organ and tissue donation programs. A major impediment to the procurement of greater numbers of transplantable organs and tissues has been the failure of health-care professionals to adequately present the next of kin the option of organ and tissue donation.<sup>9</sup> Grieving relatives of a potential donor may not spontaneously consider donation, but they should gently be offered the opportunity by persons trained to make organ and/or tissue donation presentations (“donation approach”). The donation approach is much more effective when “decoupled” from the care of the dying or deceased by having a person separate from the patient care team make the approach. This decoupling also reduces the risk of or the perception of a conflict of interest between patient care and organ and tissue recovery. The situation has improved gradually through professional education and encouragement by federal and state legislation and initiatives.

The Department of Health and Human Services Office of the Inspector General (OIG) conducted an investigation of some tissue bank practices. In a 2001 report, the OIG recommended that tissue banks make their finances public and enhance consent processes by describing potential uses of donated tissue and disclosing the bank’s relationships with other companies.<sup>13</sup> The OIG also recommended that the FDA expedite the publication of its proposed regulatory agenda for tissue banks.

Media attention to organ and tissue transplantation has increased the recognition and credibility of OPOs, as well as the public and professional support for donation. Some media inquiries, such as the *Orange County Register’s* April 2000 report entitled “The Body Brokers,” and a number of 2006 reports regarding alleged theft and forgery in tissue donation consent and recovery, may have served to shake the public’s confidence that donated tissues are handled with respect and do not result in excessive corporate profits. Such articles triggered a national debate on the ethics of procuring and processing tissue for profit. Questions were raised about the propriety of financial gain from tissue donation, possible diversion of tissue needed for lifesaving purposes to cosmetic uses, weaknesses of the consent process, and the extent to which tissue banks were monitored.

There is evidence that the industry responded to such concerns. For example, one criticism voiced was that skin needed for medically necessary reconstruction was sometimes diverted for cosmetic surgical uses, potentially creating shortages of fresh



**Table 58-3.** Infectious Diseases Reported to Have Been Transmitted by Deceased Donor Allografts

| Allografts       | Infectious Disease                       |
|------------------|------------------------------------------|
| Bone             | Hepatitis C                              |
|                  | Hepatitis, unspecified type              |
|                  | Human immunodeficiency virus, type 1     |
|                  | Bacteria                                 |
|                  | Tuberculosis                             |
| Cornea           | Fungus                                   |
|                  | Hepatitis B                              |
|                  | Rabies                                   |
|                  | Creutzfeldt-Jakob disease                |
|                  | Cytomegalovirus (?)                      |
| Dura mater       | Bacteria                                 |
|                  | Fungus                                   |
|                  | Creutzfeldt-Jakob disease                |
|                  | Hepatitis B                              |
|                  | Tuberculosis                             |
| Heart valve      | Fungus                                   |
|                  | Bacteria                                 |
|                  | Cytomegalovirus (?)                      |
|                  | Human immunodeficiency virus, type 1 (?) |
|                  | Creutzfeldt-Jakob disease                |
| Skin             | Bacteria                                 |
|                  | Cytomegalovirus (?)                      |
|                  | Human immunodeficiency virus, type 1 (?) |
|                  | Creutzfeldt-Jakob disease                |
|                  | Bacteria                                 |
| Pericardium      | Bacteria                                 |
| Pancreatic islet | Bacteria                                 |

Adapted from Eastlund.<sup>14,15</sup>

skin for burn patients. Today donated human skin is routinely recovered using a thin and thick method (thin for burn patients and thick to provide collagen), which is balanced to ensure that availability of skin for burn patients is not compromised. Additionally, research has resulted in development of clinically useful synthetic products that fill most of the needs for dermal components of products for use in cosmetic procedures.

**Tissue Transplant-Transmissible Diseases and Prevention**

**Transmissible Diseases**

Despite a careful donor selection process, the risk of donor-to-recipient transmission of viral, bacterial, fungal, and prion diseases cannot be eliminated (Table 58-3).<sup>14-16</sup> In one well-publicized case, 48 organ or tissue recipients received an organ or tissue from a single donor who, although he had no risk for HIV infection found by history, proved to have been recently infected with HIV and in the window period before HIV-1 antibody could be detected by the assays in use at the time (October 1985).<sup>17</sup> All four organ recipients became infected with HIV, but the majority of tissue recipients did not. Whole unprocessed frozen bone did transmit HIV to three recipients, while bone from which the marrow had been removed did not; transplanted

corneas, lyophilized soft tissue, and  $\gamma$ -irradiated dura mater also did not transmit the virus. This case additionally served to highlight vulnerabilities associated with inadequate disposition records, given that six recipients could not be identified from hospital records. In 2002, before routine implementation of hepatitis C virus (HCV) nucleic acid testing (NAT) for tissue donors, tissue from a man with no identifiable infectious disease risk by history or physical examination, and a negative test for anti-HCV, was found to have transmitted HCV to recipients.<sup>18</sup> All organ recipients who could subsequently be tested were found to be infected with HCV. Among 32 tissue recipients, five probable cases occurred: one of two saphenous vein recipients, one of three tendon recipients, and one of three recipients of tendon with bone allografts. All eight recipients whose infection was linked to the transplant were determined to be infected with the same HCV genotype. The current risk of viral transmission is thought to be exceedingly low.<sup>19,20</sup> In addition to donor selection and testing strategies, processing methods now in use further reduce the risk for tissues not requiring viable cells.

West Nile virus transmission linked to breastfeeding of a woman's own infant, to organ transplantation, and to blood transfusion have been reported,<sup>21</sup> but transmission through donor tissue has not been documented. Chagas' disease has been linked to transplantation of organs but transmission of parasitic diseases through vascular tissue allografts (considered likely to be possible) has not been reported.<sup>22,23</sup> Although vessel grafts associated with organ transplantation, in which arteries or veins from a different donor may be used if the donor and/or recipient vessels are damaged or insufficient, are not considered tissue by the FDA, such grafts caused documented transmission of rabies to two recipients in 2004.<sup>24</sup> Transmission of malignancy via tissue transplantation has not been reported, but is thought to be possible. Infectious diseases can also be transmitted through transplantation of tissue from living donors. Cases are well documented in the semen banking arena (Table 58-4),<sup>25</sup> as well as in the use of surgical discard bone, such as a femoral head collected during hip arthroplasty.<sup>15</sup>

Transmission of bacteria is a known risk of tissue transplantation. Bacteria can be present in the donor, either as a normal occurrence or as the result of a disease process or medical intervention.<sup>16</sup> Resuscitation efforts can increase the dispersion of such organisms. In addition, agonal bacteremia is a well-known process whereby endogenous bacteria, such as normal intestinal flora, begin to disperse throughout the body after cessation of cardiopulmonary functions, as the putrefaction process begins. This process is accelerated in persons with sepsis, rhabdomyolysis, or a cocaine overdose before death.<sup>39</sup> Bacteria can be introduced during tissue recovery; during processing, whether through cross contamination, insufficient aseptic technique, or contaminated chemicals or solutions; or even during packaging. Some tissues, including ocular tissue, skin, and semen, are inherently not sterile even if collected aseptically; fortunately, the contaminants are primarily skin contaminants with low virulence in most recipients, and are susceptible to standard antibiotics.

**Table 58-4.** Transmission of Infectious Diseases to Semen Recipients

| Reference                    | Agents and Diseases           | No. of Reported Cases/<br>No. of Women Exposed* | Transmissibility |
|------------------------------|-------------------------------|-------------------------------------------------|------------------|
| Stewart et al <sup>26</sup>  | HIV-1 (donor)                 | 4/8                                             | Yes              |
| Chiasson et al <sup>27</sup> | HIV-1 (donor)                 | 1/134                                           | Yes              |
| Rekart <sup>28</sup>         | HIV-1 (donor)                 | 2/24                                            | Yes              |
| Mascola et al <sup>29</sup>  | HIV-1 (donor)                 | 1/89                                            | Yes              |
|                              |                               | 2/46                                            | Yes              |
|                              |                               | 1/10                                            | Yes              |
| CDC <sup>30</sup>            | HIV-1 (husband-wife)          | 1/1                                             | Yes              |
| Berry et al <sup>31</sup>    | Hepatitis B virus             | 1/1                                             | Yes              |
|                              | Hepatitis C virus             | 0                                               | Likely           |
| Fiumara <sup>32</sup>        | Gonorrhea                     | 1/1                                             | Yes              |
| Hansen et al <sup>33</sup>   | Gonorrhea?                    | 3/unknown                                       | Yes              |
| Barwin <sup>34</sup>         | <i>Ureaplasma urealyticum</i> | 1/1                                             | Yes              |
| Caspi et al <sup>35</sup>    | <i>Mycoplasma hominis</i>     | 1/1                                             | Yes              |
| Kleegman <sup>36</sup>       | <i>Trichomonas vaginalis</i>  | 2/2                                             | Yes              |
| Moore et al <sup>37</sup>    | HSV-2                         | 1/2                                             | Yes              |
| Nagel et al <sup>38</sup>    | <i>Chlamydia trachomatis</i>  | 1/1                                             | Yes              |
| Kleegman <sup>36</sup>       | Group B streptococcus         | 2/2                                             | Yes              |
|                              | Cytomegalovirus               | 0                                               | Possible         |
|                              | Human papilloma virus         | 0                                               | Possible         |
|                              | HTLV-I                        | 0                                               | Likely           |
|                              | Syphilis                      | 0                                               | Possible         |

\*Number tested, where applicable.

Used with permission from Linden and Crister.<sup>25</sup>

HIV-1 = human immunodeficiency virus, type 1; HSV-2 = herpes simplex virus, type 2; HTLV-I = human T-cell lymphotropic virus, type I.

At particular risk for transmission of bacteria are the articular cartilage allografts used in knee surgery, because these cannot be subjected to extensive processing if their mechanical properties and chondrocyte viability are to be preserved (note, however, that the latter has not been proven to be essential). In 2001, a 23-year-old man was found to have died from *Clostridium sordellii* sepsis following receipt of a femoral condyle allograft. An extensive investigation identified 14 patients who had received allografts between 1998 and 2002 and who developed postoperative infections with *Clostridium* species.<sup>40</sup> The tissues were derived from nine donors, but all were prepared by the same processor, and investigation identified several factors that could have contributed to the infections. Although the tissues were cultured, they had already been suspended in antimicrobial solutions, likely leading to false-negative results. Additionally, for two of the donors, including the donor of the tissue that was implicated in the fatal case, the interval between death and refrigeration of the body (19 hours for the donor in the fatal case) exceeded the industry's voluntary standards at the time, likely permitting excessive bacterial proliferation. Tissue was processed aseptically without the use of disinfection or terminal sterilization, and the processing methods used had not been validated. Human error can also lead to release of contaminated tissues. In one case, a technician failed to follow standard procedures, resulting in release of tissue labeled as

having been subjected to irradiation when, in fact, no irradiation had occurred.<sup>41</sup> However, a wound infection in a tissue recipient, or even disseminated bacteremia, does not necessarily implicate the donor tissue. Site infections are a well-known risk of many surgical procedures and can result from the use of contaminated solutions or equipment insufficiently sterilized between procedures.<sup>42</sup>

### Risk Reduction Procedures

After death has been declared and consent for donation has been given, the process of determining the suitability of the potential deceased donor begins. The risk of disease transmission is minimized by collection and careful review of 1) information obtained during interviews with family member(s) or other knowledgeable historian(s) and health-care providers; 2) available medical records; 3) findings of a physical assessment; 4) results of an autopsy, if performed; and 5) results of blood tests for infectious disease markers. Procedures for processing and, in some cases, sterilizing tissues also contribute to tissue safety.

### Donor History Screening

Evaluation of the health and behavioral risk history of the prospective donor is an important step in establishing the suitability

of tissues for transplantation and preventing the transmission of infectious diseases. Family members may be able to provide reliable medical and behavior information, but friends and associates may provide relevant information not known to family members, especially the legal next of kin. Medical personnel may also be aware of significant medical and risk information. General donor eligibility criteria include the absence of systemic infection or any infectious or malignant disease transmissible by tissues and of behavioral risks for HIV infection or viral hepatitis. Malignancy generally disqualifies the donor, unless the malignancy is nonmetastatic or not known to metastasize to the tissue to be recovered (eg, virtually no cancer is known to metastasize to the eye or skin), and there is no suspicion of direct regional spread. The donor history review is specifically designed to reject those at high risk for viral hepatitis or HIV (eg, nonmedical injected drug users; men who have had sex with other men; persons who exchanged sex for money or drugs; persons with hemophilia or related clotting disorders; and persons with recent significant incarceration or with symptoms suggestive of a current viral infection).<sup>10</sup>

Beyond the general selection criteria for donors, there are specific eligibility criteria for each tissue type, to facilitate selection of tissues that will function adequately, as well as those that will not transmit disease. For bone and soft tissue, these include such factors as: donor age, for bone that is intended to be used for weight-bearing functions; no evidence of significant metabolic bone disease or a connective tissue disorder; and no exposure to toxic substances that could accumulate in the tissue to be recovered. Donors of cardiac tissues are screened for a history of significant valvular disease or cardiac infection, and vascular donors may be determined ineligible if there is a history of diabetes, vasculitis, varicose veins, or significant atherosclerosis. Cardiac and vascular tissue donations are also limited by age restrictions per AATB standards and individual tissue bank policy. When skin is recovered, areas of skin exhibiting signs of a skin infection, or where a rash, nevus, or tattoo is present, are avoided. Cornea donors cannot have a history of refractive corneal procedures, such as radial keratotomy. Also per EBAA and AATB standards, tissue recovery sites in deceased donors are evaluated for trauma and tissue is not recovered from sites found to be damaged. Donors of reproductive cells or tissues are screened for evidence or risk of inheritable diseases, and there are age restrictions. Extraembryonic tissue such as amnion and umbilical vein require the delivery to be full term; meconium staining of amniotic fluid is not acceptable, and there can be no current pelvic or vaginal infection in the mother. Consent is obtained from both parents.

In an effort to improve the efficacy and uniformity of the donor history acquisition process, a multiorganizational project team was established to develop a standardized donor history questionnaire and materials, modeled after the acceptable full-length donor history questionnaire and accompanying materials for blood donors. The project team includes members from the AATB, AABB, Association of Organ Procurement

Organizations, EBAA, NATCO (formerly the North American Transplant Coordinators Organization), Centers for Disease Control and Prevention (CDC), FDA, Health Resources Services Administration (HRSA), United Network for Organ Sharing (UNOS), and Health Canada. The goal is to eliminate questions that do not contribute significant information, and to develop simple and concise questions that employ terminology easily understood by the lay public. The ongoing effort is intended to produce a standardized form, with an accompanying standard operating procedure, to serve as a guide for donor history information collection by tissue banks. Use of the form is not intended to be mandatory, but it will be designed to facilitate effective history collection, in compliance with regulatory and professional standards requirements.

### Donor Physical Assessment

The physical assessment seeks evidence that is consistent with risk for infectious diseases that would disqualify the donor. Such findings include unexplained jaundice or icterus; enlarged lymph nodes; unexplained white lesions in the oral cavity; blue or purple spots on the skin (possible Kaposi sarcoma); signs of non-medical injected drug use; unexplained hepatomegaly; genital lesions consistent with a sexually transmissible disease; signs of systemic infection (generalized rash or petechiae); tattoos, piercing, or other body art that appears recent; trauma to intended retrieval sites; corneal scarring; and a rash, a scab, or a lesion that is suspicious for vaccinia. The physical assessment is fully documented so that it can be reviewed, along with medical/social/behavioral history, medical records, and records of an autopsy (if performed). AATB offers a sample tissue donor physical assessment form and instructions for its use (see <http://www.aatb.timberlakepublishing.com/files/guidancedocument1v2.pdf>), to facilitate the performance and documentation of this assessment in a thorough manner by tissue banks.

### Tissue Recovery

Tissues are collected aseptically in an operating room, in an autopsy room, or other suitable location where aseptic procedures can be performed. AATB standards require control of recovery site parameters including size/space, location, traffic, lighting, plumbing/drainage, ventilation, cleanliness of the room and furniture surfaces, presence or absence of pests, absence of other activities occurring simultaneously, sources of contamination, and capacity to permit proper handling of contaminated equipment and disposal of biohazardous waste. In addition, all working surfaces must be disinfected. Following a surgical scrub, the person performing the recovery dons proper attire (gown and gloves). Body preparation, including shaving body hair; cleansing the skin with antimicrobial agent(s); and surgical draping of the body is performed, consistent with aseptic technique. Using aseptic technique, recovery of tissue is sequenced, and well-defined zone recovery methods are employed. These recovery methods are described in AATB's Guidance Document 2, "Prevention of

Contamination and Cross-contamination at Recovery: Practices and Culture Results” (see <http://www.aatb.timberlakepublishing.com/files/bulletin46attachment1.pdf>). Generally, recovered tissues are not processed at the time of recovery. Tissues are often cultured at recovery, then individually packaged in sterile wraps, labeled with a unique donor identifier, placed on wet ice, and sent without delay to tissue banks that will process them. Following donation, the donor body is usually reconstructed to permit normal funeral arrangements and viewing. The organ/tissue donor coordinator’s responsibilities continue after the transplant procedure, in that a letter of appreciation may be sent to the next of kin, giving general information about the use of the donated organs and tissues. This communication serves as a liaison between the donor’s family and the organ/tissue procurement agency. Later, contact may be reestablished to assist in the amelioration of grief and bereavement. However, some donor families specifically request that there be no postdonation communication. AATB standards require formal establishment of donor family services. AATB has published a guidance document, “Providing Service to Tissue Donor Families,” which describes support that should be considered (see <http://www.aatb.timberlakepublishing.com/files/2007bulletin34attachment.pdf>).

### Infectious Disease Testing

Infectious disease marker testing includes HIV-1 and HIV-2 antibodies and nucleic acid, HCV antibody and nucleic acid, hepatitis B surface antigen (HBsAg), human T-cell lymphotropic virus, types I and II (HTLV-I/II) antibody, and syphilis. Tissue banks also screen donors for antibodies to hepatitis B core antigen (anti-HBc), which, although a confirmatory test is not available, may indicate HBV infection even when HBsAg is not detectable. Whenever possible, infectious disease marker testing should be performed on pretransfusion/preinfusion blood samples to avoid false-negative results caused by dilution in the event that transfusions and/or infusions had been given shortly before the time of death.<sup>43</sup> In the case of posttransfusion/infusion specimens, algorithms for determining the extent of plasma dilution are available.<sup>10</sup> Testing of postmortem (postasystole) blood specimens may be complicated by hemolysis or the presence of sediment, which can cause false-positive results in some HBsAg enzyme immunoassays (EIAs) and false-negative results in some HIV, HBV, and HCV NAT assays.<sup>19</sup> There are tests on the market for HIV and hepatitis that have been validated for use with postmortem specimens and approved by the FDA for use in tissue donor screening (see <http://www.fda.gov/cber/tissue/prod.htm>). When practical, tissue from living donors is preserved and quarantined before the donor is retested for HIV and hepatitis viruses in order to rule out seroconversion during the period of storage. Specific regulatory requirements for quarantine apply to donor semen. Additionally, both semen and oocyte donors must be found negative for *Neisseria gonorrhea* and *Chlamydia trachomatis* and are usually tested for carriage of at least one genetic disorder, as indicated by donor racial and ethnic

background. Donors may also undergo specific testing for rare genetic disorders if the recipient couple seeks a donor known to be negative for a particular gene mutation.

### Tissue Sterilization

Tissue sterilization is defined as the killing or elimination of all microorganisms, whereas disinfection refers to the removal of microbial contamination, from allograft tissue. The Association for the Advancement of Medical Instrumentation (AAMI), a standard-setting organization for the medical manufacturing industry, defines Sterility Assurance Level (SAL) as the probability that an individual device, dose, or unit is nonsterile (ie, one or more viable microorganisms being present) after it has been exposed to a validated sterilization process. While absolute sterility theoretically would represent an absence of any pathogen, SAL is generally applied only to the level of possible contamination with bacteria or parasites. In contrast to log reduction of viruses determined in assessments of virus reduction methods, SAL is an absolute determined by the ability of the method to eradicate or reduce microorganisms, the susceptibility of organisms that may be present to the sterilization method applied, and the maximal bioburden that could occur in the initial material. Thus, a process validated to achieve a specific SAL must include a step to assess the initial bioburden present and its susceptibility in order to qualify a specific level of assurance. For example, an SAL of  $10^{-6}$  means that there is less than a 1 in 1,000,000 chance of a viable microorganism remaining after the sterilization procedure. The FDA requires that medical devices be sterilized using a method validated to achieve an SAL of  $10^{-6}$ . A medical device derived from or that includes a biological product component must also meet an SAL of  $10^{-6}$  if it is to be labeled sterile. An SAL of  $10^{-3}$ , or a 1 in 1000 chance of a viable microorganism being present, is a more achievable goal selected by some processors for aseptically processed tissues unable to withstand the harsh treatment needed to achieve a more restrictive SAL without an impairment of tissue function. Such tissues may not then be labeled as sterile.

The complex physical structures and density of musculoskeletal tissues pose challenges for adequate penetration of antimicrobial agents and eradication of microorganisms. Allografts will not tolerate methods usually applied to metal and plastic medical devices because such treatment would impair the mechanical and biologic properties necessary for clinical utility. As an alternative, sterilization of tissues has been accomplished by several methods, including heat, chemicals, ethylene oxide gas, and irradiation. However, not all sterilants have adequate tissue penetration. This is particularly the case for gases and liquids. The initial bioburden, which may be high in some tissues, must be considered. Some tissues are treated with antibiotics in vitro before storage, but this treatment decontaminates only the surface and may be effective against bacteria only.

A variety of methods, including chemical treatments and irradiation, have been used to reduce or eliminate pathogens in

tissue intended for transplantation. The introduction of bone sterilization by ethylene oxide gas simplified bone processing and facilitated the widespread use of sterilized air-dried and lyophilized bone products.<sup>44</sup> The effects of ethylene oxide treatment on the biomechanical and osteoinductive capacity of bone allografts have been questioned, although animal studies have yielded inconsistent results.<sup>45-47</sup> These concerns, combined with those regarding the carcinogenic potential of ethylene oxide and its breakdown products, have largely led to abandonment of this method in the United States and the United Kingdom.

First introduced over 30 years ago,  $\gamma$ -irradiation of bone is still used widely, usually employing a cobalt-60 source. The  $\gamma$ -rays penetrate bone effectively and work by generating free radicals, which have adverse effects on collagen and limit utility in soft tissues unless performed in a controlled dose fashion at ultra low temperature. The minimal bacteriocidal level of  $\gamma$ -irradiation is 10 to 20 kGy (1 kGy = 100,000 rad). Uncontrolled human studies have shown irradiated, calcified, and demineralized bone grafts to be clinically effective.<sup>48,49</sup> Numerous studies have shown that mineralized bone allografts irradiated at 25 to 30 kGy are also clinically effective, with success rates of 85% to 91% reported.<sup>50,51</sup> In controlled studies, the clinical effectiveness of bone allografts subjected to 25 kGy irradiation was comparable to that of nonirradiated bone grafts,<sup>52</sup> although doses exceeding 25 kGy for cortical bone and 60 kGy for cancellous bone have been found to induce cross-linking of collagen and to impair mechanical function in a dose-dependent fashion.<sup>53</sup> There is in-vitro evidence that irradiation reduces osteoclast activity and increases osteoblast apoptosis, and that residual bacterial products induce inflammatory bone resorption following macrophage inactivation.<sup>53</sup> However, the clinical significance of these findings has not been established. Newer processes employing radioprotectants have preserved bone allograft integrity when doses >25 kGy are applied and controlled-dose methods permit successful irradiation at lower doses (see proprietary chemical sterilization methods to follow). Irradiated demineralized bone has active osteoinductive activity and has been effective in non-structural clinical applications.

Concerns about pathogen transmission and the limitations of irradiation, especially for soft tissues, have prompted improvements in sterilization methods and in the validation of these methods. A number of proprietary chemical-based processing methods have been developed with aims of effectively penetrating tissues and reducing, killing, or inactivating microorganisms and viruses without unacceptable adverse effects on the tissue's biomechanical properties. Additionally, for use in transplantation, the agents must either be able to be effectively removed or be nontoxic. All methods in current use are applied only to tissue from donors who have met stringent criteria for medical history and behavioral risk assessment as well as negative results on infectious disease marker testing. Some popular methods used are described below.

The Tutoplast process (Tutogen Medical, Gainesville, FL) was the first comprehensive method to sterilize and preserve

tissue without compromising its biological or mechanical properties. The process has been in use for over 30 years, for a variety of hard and soft tissues, including bone, fascia lata, pericardium, dermis, amniotic membrane, and sclera. Initially, lipids are removed in an ultrasonic acetone bath that also inactivates enveloped viruses and reduces prion activity. Bacteria are destroyed using alternating hyperosmotic saline and purified waterbaths that also wash out cellular debris. Soluble proteins, nonenveloped viruses, and bacterial spores are destroyed in multiple hydrogen peroxide baths, and a 1N sodium hydroxide treatment further reduces prion infectivity by 6 logs. A final acetone wash removes any residual prions and inactivates any remaining enveloped viruses. Vacuum extraction dehydrates the tissue before the grafts are shaped and then double-barrier packaged. Terminal sterilization using low-dose  $\gamma$ -irradiation yields an SAL of  $10^{-6}$ .

The Allowash XG process (LifeNet Health, Virginia Beach, VA) employs six steps, including bioburden control and assessment; minimization of contamination during processing; rigorous cleaning and disinfection steps, which employ detergents, antibiotics, alcohols, and hydrogen peroxide; and a final step of low-temperature, controlled-dose  $\gamma$ -irradiation. The process has been validated to achieve an SAL of  $10^{-6}$ .

The BioCleanse process (Regeneration Technologies, Alachua, FL) employs low-temperature addition of chemical sterilants, such as hydrogen peroxide and isopropyl alcohol, which permeate the tissue's inner matrix, followed by pressure variations intended to drive the sterilants into and out of the tissue. Regeneration Technologies reports an SAL of  $10^{-6}$  for soft tissues without adverse effects on the initial allograft mechanical properties.

The Clearant process (Clearant, Los Angeles, CA) avoids the negative effects of  $\gamma$ -irradiation through addition of free radical scavengers, employing dimethylsulfoxide (DMSO) and propylene glycol as pretreatment radioprotectants. Although the process subjects tissue to 50 kGy of radiation and achieves an SAL of  $10^{-6}$  for bacteria, fungi, yeast, and spores, the tissue's biomechanical properties are retained.

The Musculoskeletal Transplant Foundation (Edison, NJ) uses a series of chemicals, including nonionic detergents, hydrogen peroxide, and alcohol, to treat cortical and cancellous bone grafts. For soft tissues, such as bone-patellar tendon allografts, an antibiotic mixture containing gentamicin, amphotericin B, and primaxin is added, and then washed out to a nondetectable concentration. The Musculoskeletal Transplant Foundation claims an SAL of  $10^{-3}$  for its products. Incoming tissues whose bioburden exceeds prescribed parameters are pretreated with low-dose  $\gamma$ -irradiation.

NovaSterilis (Lansing, NY) has developed a sterilization technique that uses supercritical carbon dioxide at low temperatures and relatively low pressures, resulting in transient acidification, which is lethal to bacteria and viruses, with good penetration reported. However, this technique is still in development, and data on retention of allograft mechanical properties are limited.

## General Principles of Tissue Preservation and Clinical Use

Except for bone, which is usually lyophilized (freeze dried), the most common method of storing recovered tissues is at cold temperatures, either by refrigeration or by freezing (Table 58-5). For short-term storage, refrigeration at about 4°C often suffices, whereas long-term storage usually requires a frozen state. Several types of tissues can be preserved by multiple methods. Bone, dura mater, and amnion can be effectively cryopreserved or lyophilized. Much of the lyophilized and cryopreserved human tissue used in transplantation is intended to serve a structural purpose and maintenance of cell viability is not necessary. Tissues of this type, which include dermis, are composed of an extracellular matrix (such as collagen) with few or no viable cells present to support the matrix after transplantation, although they can contribute growth factors to facilitate remodeling. Even when the processing method used is intended to preserve cell viability, the donor cells will die following transplantation. The extracellular matrix, whether transplanted containing viable cells or devoid of them, is repopulated through the ingrowth of metabolically active recipient cells. More gradually, depending in part on the size and type of the allograft implanted, remodeling occurs, and the transplanted structure may eventually be entirely replaced by host cells.<sup>55</sup> The transplantation of allograft heart valves and cardiac conduit tissues provided in an acellular matrix form is being studied to determine the rate and extent of repopulation with recipient cells.<sup>56</sup>

In some tissues, such as cornea, a single layer of viable donor cells is important, and this requirement necessitates maintenance of the tissue in culture medium at refrigerated temperature.<sup>57</sup>

Other human tissues, such as marrow, skin, and gametes, are stored by either refrigeration or cryopreservation. In the latter, a controlled-rate freezing process and a cryoprotectant remove water from the cells while maintaining viability. The usefulness of tissues requiring posttransplantation cell viability depends on their maintenance of not only metabolic activity, but also capacity to synthesize protein, proliferate, or differentiate.

### Bone

Bone allografts have many uses, including provision of acetabular and proximal femoral support for replacement of failed prosthetic hip joints, packing of benign bone cysts, fusion of the cervical or lumbar spine to correct disk disease or scoliosis, restoration of alveolar bone in periodontal pockets, reconstruction of maxillofacial deficits, and replacement of bone that has been resected because of a bone malignancy, such as osteosarcoma (see Table 58-1). The last procedure is accomplished with large osteochondral allografts that permit tumor resection and achievement of a cure without limb amputation.

Historically, bone allografts were prepared by cutting a larger graft using simple techniques. Today's technology allows the cutting, machining, and piecing together of allografts via precision instrumentation, and has resulted in stronger and more versatile grafts that can withstand the challenges of new surgical techniques. Linear grooves, notches, or crosshatchings may be incised into bone surfaces to make the bone graft less likely to slip or become dislodged after placement. Many bone allografts, especially those used in neurosurgical applications, are now placed using precision instrumentation that not only ensures exact placement, but also enhances stability. Allografts can be cut or shaped

**Table 58-5.** Recommended Human Tissue Storage Conditions and Durations

| Tissue                   | Storage Condition                | Maximal Storage Duration |
|--------------------------|----------------------------------|--------------------------|
| Bone                     | −40°C or colder                  | 5 years*                 |
|                          | −40 to −20°C                     | 6 months                 |
|                          | 1–10°C                           | 5 days                   |
| Tendon                   | −40°C or colder                  | 5 years*                 |
| Fascia lata              | Lyophilized, ambient temperature | 5 years*                 |
|                          | −40°C or colder                  | 5 years*                 |
| Articular cartilage      | −40°C or colder                  | 5 years*                 |
|                          | 1–10°C                           | 5 days                   |
| Skin                     | −40°C or colder                  | Not defined              |
|                          | Lyophilized, ambient temperature | Not defined              |
| Cornea                   | 2–8°C                            | 14 days                  |
| Semen                    | Liquid nitrogen, immersed        | Not defined              |
|                          | Liquid nitrogen, vapor phase     | Not defined              |
| Cardiac, vascular tissue | −100°C or colder                 | Not defined              |
| Dura mater               | Lyophilized, ambient temperature | Not defined              |

\*Or as validated by the processor.

Adapted with permission from Pearson et al<sup>10</sup> and Eastlund.<sup>54</sup>

to precise angles that accommodate, for instance, lordosis of the cervical and lumbar spine. Advanced processing methods have resulted in improvements in availability and also in graft biocompatibility and functioning, in such processes as osteoinduction, osteoconduction, bioresorption, and tissue regeneration.<sup>1</sup>

Fresh autograft can be taken from the patient's own iliac crest during surgery, but this practice is becoming less common.<sup>55</sup> Fresh bone autograft is preferred by some surgeons, but preserved allografts are practical and accepted alternatives that approximate the results obtained with fresh bone autograft.<sup>58</sup> In some patients, an autograft is not an option because sufficient bone is not available. In addition, the use of bone allografts reduces operating room time and the number of operative sites, leading to reduced morbidity and lower cost. The use of bone allograft does carry the risk of donor-to-recipient transmission of infectious disease,<sup>15</sup> although this risk is minimized through careful donor selection and testing, and disinfection and sterilization of tissue during processing.

### Frozen Bone

Frozen bone, collected under aseptic conditions and then frozen or cryopreserved, is available in a wide variety of shapes and sizes from deceased donors, or as femoral heads or tibial plateaus obtained from living donors undergoing total joint replacement in an operating room (now an uncommon practice in the United States, but continuing in Canada, Australia, and Europe). This frozen bone is largely free of bacteria, but it does carry the risk of viral transmission. Diseases known to have been transmitted by unprocessed bone include AIDS, tuberculosis, and hepatitis.<sup>15</sup>

Frozen bone can cause alloimmunization from exposure to antigens on the attached connective tissues, marrow, and blood, although such alloimmunization apparently does not affect the graft's efficacy. Detailed reviews addressing the role of histocompatibility and the immune response in bone allograft transplantation have been published.<sup>59,60</sup> Antibodies to histocompatibility antigens,<sup>59,60</sup> blood group antigens,<sup>61,62</sup> and bone matrix proteins have been induced by transplanted frozen bone. In order to avoid Rh alloimmunization, bone from an Rh-negative donor is usually selected when using bone that has not been processed to remove red cells and the recipient is an Rh-negative female of childbearing potential.

Bone that otherwise would be discarded can be collected from living donors during surgical procedures (total hip or knee replacements with prostheses). The eligibility of a volunteer living donor is determined by a careful health history review, a directed physical examination if indicated, and donor testing for anti-HIV-1 and -2, HIV-1 NAT, HBsAg, anti-HBc (total), anti-HTLV-I/II, anti-HCV, HCV NAT, and syphilis.<sup>10</sup> In some countries, if NAT assays are not performed, the donated tissue is quarantined, and the living donor is retested 6 months later for infectious disease markers. This quarantine and retesting process is intended to eliminate donors who were in the early stage of viral infection but were antibody negative at the time of donation (in the window period). A follow-up anti-HBc test may

detect donors who had been infected with HBV shortly before donating but who no longer have detectable HBsAg.

Generally, culturing for bacteria is performed on each bone to be fresh frozen or on each batch/lot of cryopreserved bone. Allograft bone is further prepared by removal of extraneous tissue; it is then packaged, and immediately either shipped to the processing tissue bank on wet ice or sent to temporary freezer storage at  $-40^{\circ}\text{C}$  or colder. Frozen bone allografts are available as whole bones or cut into usable shapes and sizes. Frozen bone can generally be stored up to 5 years at  $-40^{\circ}\text{C}$  or colder, but the maximal storage duration and expiration date may vary based on processing and storage methods, as validated by the tissue bank. There is no evidence that the biomechanical or osteoinductive properties decline during frozen storage. However, in the absence of cryopreservation, frozen bone does not maintain cellular viability. Thus, frozen bone is used for structural support that depends on an intact calcified extracellular matrix or is used as filler to promote new bone formation.

### Lyophilized Bone

Following aseptic recovery, deceased donor bone can be placed on ice for transport to temporary storage in a freezer and maintained frozen at  $-40^{\circ}\text{C}$  or colder, and then can later be sent to a tissue processor with dry ice as a refrigerant. Alternatively, immediately after recovery, the tissue can be placed on wet ice and expedited directly to the processing tissue bank where, within 72 hours of recovery, it is frozen at  $-40^{\circ}\text{C}$  or colder until processing. Such processing includes removal of surface tissues and internal fat, blood, and marrow by means of mechanical agitation, high-pressure water jets, and alcohol soaks. It can also include detergents and other solutions as part of a proprietary process. Then the bone is milled into clinically useful shapes and sizes. This may include computer-guided milling and use of assemblies that result in complex mechanical structures. Conventional allografts include corticocancellous strips, wedges, and dowels; cortical struts and rings; and cancellous and corticocancellous cubes and chips. Bone can also be ground into a powder and be available as DBM and products that include it. DBM, which is also known as demineralized freeze-dried bone allograft, is derived from cortical bone and is available in combination products in the form of gels, pastes, putties, and flexible strips or sheets. DBM itself may be obtained in specific granule or particle sizes, as a powder, or in entangled, twisted fiber configurations. DBM primarily provides growth factors, but accompanying collagen can help play a structural role as a scaffold for future bone growth. The combining of DBM with approved polymer carriers results in moldable grafts that are user friendly for the surgeon, do not migrate after placement, and whose bone content does not dissolve following transplantation. Such grafts can readily be applied to completely fill bony defects and to act as a scaffold for ingrowth of the recipient's own cells, or they can be used to enhance other structural repair devices, such as dental implants, vertebral body spacers and cages, or support devices such as rods, screws, and plates.<sup>1</sup>

The bone allografts are lyophilized to a residual moisture content of <6% or 8% (depending on measurement method) and packaged into peel packs or “boat” packaging. Routine quality control testing of bone is designed to monitor safety, rather than potency or efficacy. Sterility is assessed by the testing of samples of each batch, or by another acceptable method. If a robust validated sterilization process is used, sterility may need to be assessed only at established intervals, such as quarterly. Potency can be evaluated by using assays for osteoinductive capacity and biomechanical properties, but these analyses usually are conducted only when there is a change in the production process. Lyophilized bone is brittle unless fully rehydrated before use. Lyophilized bone can usually be stored at ambient temperature for up to 5 years if the graft’s package integrity and its vacuum are maintained, depending on validation performed by the tissue processor.

Bone collected aseptically in an operating room and processed aseptically can be lyophilized without use of a sterilant. Because the bone, theoretically, is bacteria-free, final sterilization with  $\gamma$ -irradiation may not be necessary. Although the bone should be free of bacteria, it still has the potential to transmit disease. Despite this risk, some physicians have preferred aseptically processed, nonsterilized lyophilized bone because it was thought to have better osteoinductive capacity than sterilized bone. However, controlled-dose low-temperature radiation has been found to have no significant effect on osteoinductive capacity.<sup>63</sup>

### Ear Ossicles

Ear ossicles are used as a special kind of bone graft to correct selected cases of deafness in which the patient’s own ossicles have suffered congenital, traumatic, or postinfectious damage.<sup>64</sup> Ear ossicles are procured by removal of the temporal bone en bloc or as a core with a bone-plug cutter. The temporal bone can be stored temporarily, for months if frozen, or up to 2 weeks if preserved in formalin; the tympanic membrane and ossicular chain are then dissected. Ossicles have been stored for up to 2 months in cialit (an organomercuric compound), and for up to 1 year at room temperature in buffered formaldehyde. Alternatively, ossicles are dissected at the time of collection, lyophilized, and then sterilized by  $\gamma$ -irradiation. Lyophilized ossicles can be stored at ambient temperature for up to 5 years.

### Connective Tissue

#### Cartilage and Meniscus

Human cartilage can be transplanted at weight-bearing or non-weight-bearing sites. For non-weight-bearing uses such as nasal reconstruction and mandibular or orbital rim augmentation, the graft provides structural support and need not be viable. Costal cartilage can be recovered for this use. The cartilage can be sterilized by  $\gamma$ -irradiation and stored in saline at refrigerated temperatures, or it can be lyophilized and stored at ambient temperature.

Articular cartilage can be transplanted to weight-bearing articular surfaces to replace focal cartilage defects caused by

trauma or degenerative disease, particularly in the knee. Cartilage in an osteochondral or osteoarticular allograft can be obtained as a femoral hemicondyle, a tibial plateau or fragment, or a measured segment removed with a template cutter that can be press-fitted into a similarly cut area in the recipient. Osteochondral allografts avoid autograft harvest site morbidity and are advantageous when the focal articular cartilage defects being repaired are large (>2.5 cm).<sup>65</sup> It has been assumed that, in weight-bearing applications, chondrocytes must survive the collection and preservation process and remain viable, producing normal cartilage matrix to maintain mechanical properties. It appears that chondrocytes deep within the cartilage matrix resist cell-mediated immune responses by the recipient and, if kept viable during storage, are able to survive after transplantation. Cartilage grafts from histoincompatible donors, stored <24 hours at 4°C, have survived for as long as 7 years after transplantation, if the grafts developed a sound union and if conditions for correct biomechanical functioning were present.<sup>66</sup> Articular cartilage collected in a sterile manner can be stored at 4°C in saline or electrolyte solutions with or without 10% fetal calf serum and antibiotics.<sup>67</sup> An FDA rule proposed in January 2007 would prohibit the use of certain cattle materials. This rule indicates that the FDA considers cow-to-calf transmission of bovine spongiform encephalopathy unproven by current evidence, and the agency believes that procedures could provide assurance that contamination has been prevented.<sup>68</sup> Osteoarticular and osteochondral allografts can be stored refrigerated for up to 28 days with successful clinical outcomes. If they have been cryopreserved, expiry for these allografts may be extended to 1 year. Cartilage cryopreserved with or without DMSO maintains viability with variable degrees of success.<sup>69</sup>

The use of a large osteochondral allograft, such as the femur with the articular cartilage attached, is thought to require preservation of cartilage viability in order to maintain biomechanical properties. To accomplish this, grafts have been stored at refrigerated temperatures in electrolyte solutions for up to 1 month, or have been frozen in 10% glycerol or 15% DMSO and stored at  $-70^{\circ}\text{C}$  or colder.<sup>70</sup> Following transplantation in humans and animals, the surface of the articular cartilage allograft undergoes degenerative changes within a few years. These grafts have the same risk of disease transmission as other fresh tissue allografts.

Menisci are C-shaped disks of fibrocartilage interposed between the femoral condyle and tibia. The presence and integrity of the meniscus are essential for knee mechanics and biochemical functions. Loss or disruption of the meniscus is associated with pain, joint laxity, and degenerative arthritis. Meniscal transplantation has been proposed as a method of providing a biologically and biomechanically acceptable structure to replace a damaged or removed meniscus, with a goal of relieving pain, decreasing stress on the anterior cruciate ligament, and preventing late arthritis, although evidence of allograft tissue being chondroprotective is lacking. Although there have been unpublished reports of successful transplantation of menisci stored <24 hours at 4°C, fresh menisci are not usually



available. Cryopreserved menisci are used successfully, with good outcomes (including reduced pain and increased knee function) reported.<sup>71,72</sup>

### **Tendon and Ligament**

The knee is the joint most frequently involved in sports-related injury. Arthroscopic methods for replacing the anterior or posterior cruciate ligaments with autografts, allografts, or artificial tendons and ligaments are frequently used. Despite the attendant need for sacrifice or weakening of normal structures, the use of autografts appears to have the highest success rate and the lowest incidence of complications. However, allografts may be indicated for multiple ligament knee injuries, anterior cruciate ligament revisions, or posterior cruciate ligament reconstruction, and when extensor mechanisms are impaired (as with previous tendon tears). It is sometimes preferable to avoid the morbidity associated with autograft harvesting. In addition, there are occasions when sources of adequate autograft tissue are not available.<sup>73</sup> Allografts used to replace the injured anterior cruciate ligament are usually derived from deceased donor patellar ligaments, tendons of the leg (eg, tibialis, semitendinosus, gracilis, and peroneus longus), or Achilles tendons. Ligament and tendon allografts are usually stored frozen, but some are stored lyophilized. In-vitro biomechanical properties of tendons do not seem to be greatly affected by freezing, lyophilizing, or ethylene oxide sterilization.<sup>74</sup> However, many surgeons eschew lyophilized tendon allografts because of experiences with clinical failure. Frozen tendon allografts are commonly sterilized by  $\gamma$ -irradiation, although this can reduce their mechanical strength, particularly if the dose exceeds 20 kGy.<sup>75</sup> There is no evidence that maintenance of cellular viability during processing and storage is important to clinical effectiveness. The effect of irradiation on the biomechanical properties of human tissue has been explored extensively, with inconsistent results. This is probably because the studies failed to use uniform irradiation methods and comparable study designs. A key study found a difference in average stress at failure between nonirradiated and  $\gamma$ -irradiated tendons; that difference is likely a consequence of the free radicals generated, which can cause minor crosslinking of collagen fibers and alteration of the tendon's material properties.<sup>76</sup> In order to eliminate the potential for elongation of irradiated grafts after implantation, the authors encouraged pretensioning of grafts before insertion.

### **Fascia Lata**

The fascia lata is a broad fibrous membrane surrounding the thigh muscles. The thick lateral portion acts as a flattened tendon, and its muscular insertions helping to maintain the trunk in an erect posture. Fascia lata can be removed and transplanted as an autograft or allograft. As an allograft, fascia lata has been used to suspend the upper eyelid to correct ptosis, to replace injured anterior cruciate ligaments, to provide support for bladder suspension, and to repair ankle, hip, and shoulder suspensions (ie, repair of a ruptured shoulder rotator cuff). Fascia lata usually is preserved by lyophilization, resulting in a residual moisture of <6% or 8% (depending on measurement method), the graft

is then sterilized by  $\gamma$ -irradiation and stored for up to 5 years at ambient temperature. After rehydration, the graft's biomechanical properties equal those of fresh frozen fascia lata. The use of fascia lata has become less popular because of the availability of alternative products, such as decellularized skin.

### **Dura Mater**

Dura mater is the outermost, toughest, and most fibrous of the three meningeal membranes covering the brain and spinal cord. The intracranial portion is collected, processed, stored, and distributed for several clinical applications; the most common use is the closure of dural defects caused by resection of tumor or the repair of traumatic injury. Human dura allograft most commonly is preserved by lyophilization. Ethylene oxide and  $\gamma$ -irradiation are effective in preventing transmission of viruses and bacteria; however, Creutzfeldt-Jakob disease (CJD) has been transmitted by dura mater treated by these methods. Following findings by Brown and coworkers,<sup>77,78</sup> in 1986 The Committee on Health Care Issues of the American Neurological Association recommended using 1N NaOH for 1 hour or steam autoclaving for 1 hour at 132°C as standard sterilization procedures for CJD-infected tissue or contaminated materials. Donors with a history of clinical dementia or other central nervous system disorders are not accepted as dura mater donors. Lyophilization and sterilization treatments do not lessen the effectiveness of dura mater allografts. Reconstituted freeze-dried dura mater is thick and strong, holds suture well, and is incorporated into normal surrounding tissue without rejection. Because of the risks and resulting decreased demand, dura mater is currently processed in the US by only one tissue bank.

### **Skin**

Human skin allograft is the dressing of choice for temporary grafting onto deep burn wounds whenever sufficient amounts of autograft skin are unavailable. Early excision of burned tissue and covering of the wound with deceased donor skin allograft has shortened hospitalization and decreased mortality more than has any other treatment.<sup>79</sup> A skin allograft provides temporary coverage and acts as a barrier against loss of water, electrolytes, protein, and heat. It reduces opportunities for the invasion of bacteria and speeds re-epithelialization. Skin allografts are replaced periodically until the patient's vascular bed is reestablished. Skin allografts also are used for unhealed skin defects (decubitus ulcers, autograft skin donor sites, pedicle flap donor sites, and traumatically denuded areas). While skin has historically been used only as a covering, decellularized (mechanically and chemically treated) skin offers the opportunities for use of a collagen matrix that can be implanted and be remodeled within the site with the recipient's own cells. It is used for such applications as bladder suspension surgery and repair of large defects, such as postoperative hernias and dehiscence wounds. In an analogous fashion, Taylor and colleagues<sup>56</sup> have used perfusion-decellularized cardiac tissue matrix in an attempt to develop a bioartificial heart.

After collection, fresh skin can be stored in medium at 1 to 10°C for up to 14 days,<sup>10</sup> but fresh skin is seldom used today. Skin also can be frozen using a method that retains cell viability, in order to improve availability. Because cell viability declines during refrigerated storage, results are best when cryopreservation is performed within 2 to 3 days after recovery. Cryopreserved skin can be prepared as strips (often 3-inch by 8-inch sections), either unmeshed or meshed (most commonly with a 1:1.5 expansion ratio, which triples the area that can be covered). The skin then covered in fine-mesh gauze and laid flat, is packaged, and is then cryopreserved, with glycerol or DMSO at a concentration of 10% or 15% as a cryoprotectant. Cryogenic damage is minimized by controlling the rate of freezing to between  $-1$  and  $-5^{\circ}\text{C}/\text{minute}$ . Many tissue banks use a “heat sink” freezing method, rather than one that employs computer-controlled freezing chambers. Heat sinks involve aluminum plates combined with styrofoam-insulated boxes; these are placed directly into a  $-70^{\circ}\text{C}$  mechanical freezer. This simple process provides a slow, controlled freezing rate that is acceptable for skin and that also maintains cellular viability.<sup>80</sup> AATB standards permit frozen storage in a mechanical freezer at  $-40^{\circ}\text{C}$  or colder, in the vapor phase of liquid nitrogen, or submerged in liquid nitrogen.<sup>10</sup> The maximal allowable storage period in the frozen state during which viability and structural integrity are maintained has not been determined. Cryopreserved skin allograft usually is transported from the tissue bank to the hospital with dry ice in order to maintain a frozen environment until use.

Skin for use in burn applications generally is not preserved by lyophilization because this method decreases clinical efficacy. However, lyophilized skin is sometimes used by oral surgeons to cover oral mucous membrane defects and to speed re-epithelialization. Lyophilized acellular dermal matrix is also available, in two thicknesses for different applications, and can serve as a natural biological matrix for soft tissue augmentation in soft tissue defects and in periodontal peri-implant soft tissue management. Following hydration, lyophilized skin has multidirectional strength and can adapt to surface contours, and it then is resorbed over 4 to 6 months, depending on the site, defect size, patient age and health status, and the biomechanical load on the graft. Depending on processing method and packaging configuration, lyophilized skin can be stored as long as 5 years at ambient temperature or it may require refrigeration.

### Ocular Tissue

Cornea is one of the most frequently transplanted tissues; >34,000 corneas were transplanted in the United States in 2006. Corneal transplantation has become highly effective because of improvements in suture materials, surgical instruments, and topical antiinflammatory medications to control rejection. It is now considered a standard therapy for a variety of conditions. However, early in this century demand decreased because of improvements in cataract surgery techniques compared with those in use in the 1990s, during which time use for complications of cataract surgery had supplanted keratoconus as the

leading indication. More recently, development of a new technique that uses an air bubble to hold the allograft in place, obviating the need for sutures and retaining the normal topography, has resulted in faster recovery and improved visual acuity, as well as a reduction in the occurrence of adverse effects. As a consequence, corneal transplantation for Fuchs epithelial dystrophy is becoming an accepted therapy at much earlier stages of the disease, resulting in a resurgence of demand for cornea allografts. Currently the most common indications for corneal transplantation are postcataract surgery corneal edema, keratoconus, Fuchs dystrophy, and corneal regrafting. Donor cells in the avascular full-thickness cornea graft enjoy long-term survival without the aid of histocompatibility matching because the recipient site is also almost completely avascular. Some experts believe that the failure rate of 5% to 10% might be improved by HLA matching; recipients known to be sensitized to HLA antigens have rejection rates higher than nonsensitized recipients.<sup>81</sup> The possibility of alloimmunization is of particular concern in patients who are undergoing repeat grafting procedures because of graft failure or who have ocular infections, as the corneal rim may become quite vascularized. Systemic immunosuppressants are not used, but topical corticosteroids are used routinely. Sclera may be used in the repair of ocular deficits, in orbital reconstruction following enucleation, and in some dental applications.<sup>82</sup>

Ocular tissue can be recovered by enucleation or by in situ excision of the cornea, with a rim of sclera. It is preferable that recovery be performed within 6 hours after death. The oldest method of storage for whole globes was at  $4^{\circ}\text{C}$  in a moist chamber; this method appeared to maintain viable endothelial cells sufficient for graft efficacy for as long as 48 hours after recovery. Because it yields improved viability, a more common method today is storage of the cornea, with attached rim of sclera, at  $4^{\circ}\text{C}$  in a modified tissue culture medium, based on that developed in 1974 by McCarey and Kaufman.<sup>83</sup> One example commonly used is Optisol-GS (Bausch & Lomb, Irvine, CA), which contains dextran (as an osmotic agent), chondroitin sulfate, gentamicin, and streptomycin. Storage of corneas in the medium, at 2 to  $8^{\circ}\text{C}$ , can maintain endothelial viability for as long as 14 days, and can maintain functional integrity for eutopic graft applications not requiring visual acuity for even longer storage periods.<sup>84</sup> Grafts are usually used within 7 days. Although they have been treated with antibiotics, allograft corneas are not considered sterile. An alternative culture technique, used at a few eye banks, has maintained viable corneas for as long as 5 weeks in tissue culture medium at  $34^{\circ}\text{C}$ .<sup>85</sup> Rarely, corneas are frozen with cryoprotectants. Sclera is usually preserved in  $\geq 70\%$  ethyl alcohol; such a method yields a shelf-life as long as 2 years.

### Cardiovascular Tissue

Cardiovascular tissue includes cardiac tissues (heart valves) and vessels that can be used as conduits. Donor medical history requirements differ, so AATB has established separate standards for cardiac tissues and for vascular tissues.<sup>10</sup> Since their introduction 4 decades ago, human heart valve allografts have been

shown to be a superior alternative for patients needing heart valve replacement and for whom mechanical and porcine valves are contraindicated. Human heart valve allografts do not require recipient anticoagulation, have a lower incidence of thromboembolism, and appear relatively resistant to infection. After valve allograft transplantation, donor endothelium is not maintained, but donor fibroblasts remain for an undetermined period. Because anticoagulation is unnecessary, human valve allografts are the graft of choice for children, females of childbearing potential, and patients with infection in the aortic root. The use of allograft valves has been slowed, however, because implantation is more difficult technically and because their availability is limited. Additionally, clinical results with transplantation of xenograft tissue valves have improved, although these are not available in the small sizes required by many pediatric patients.

Technical impediments have made it impossible to successfully produce man-made (either completely artificial or modified porcine) replacement heart valves for use in neonates and other pediatric patients who require very small grafts. Only donated human heart valves from newborns or small children offer unobstructed blood flow through such a small annulus. Also, the tissue's pliability renders human allografts adaptable to the ingenuity of cardiothoracic surgeons who repair congenital defects by using allografts to replace underdeveloped or otherwise defective valves or outflow tracts, or to construct valves and tracts that may be absent.<sup>86</sup> Complex repairs may need to be staged over many years or may be only palliative. Clinical use of nonvalved conduit sections of cardiac allografts, mostly from the main pulmonary artery and/or its branches, has reached a level equivalent to that of the two semilunar valve allografts. On a global scale, availability of cryopreserved pediatric allograft heart valves has historically been low and unable to meet demands.

To obtain valve allografts, hearts are recovered aseptically, immersed in a sterile isotonic solution within a sterile container, placed on wet ice, and transported expeditiously to a tissue processing facility. The pulmonic and aortic valves, along with their intact outflow tracts and/or small pieces of these conduits, are dissected free of the heart within 48 hours of donor asystole, and then placed in tissue culture medium amended with a low-dose antibiotic cocktail. Initially, human heart valves were stored up until use at 5°C in tissue culture medium containing antibiotics. However, although their cell viability was lost under these conditions after 48 hours, such grafts could be used successfully after a few weeks in such refrigerated storage. Subsequently, studies demonstrated that cryopreservation of heart valves allowed successful banking of valves of various sizes and types. Additionally, such allograft valves were found to be superior, based on their retaining the cellular matrix and having a low clinical incidence of valve degeneration, rupture, leaflet perforation, and valve-related death. For these reasons, human heart valves generally are cryopreserved,<sup>87</sup> with a method that includes an initial exposure to antibiotic solutions for 12 to 24 hours. Cryopreservation then follows, using a 10% DMSO solution tissue culture medium that often is amended with 10% fetal

calf serum. Freezing is accomplished using a computer-assisted controlled rate of  $-1^{\circ}\text{C}/\text{minute}$  to  $-40^{\circ}\text{C}$ . Valves generally are stored in the vapor phase of liquid nitrogen. Heart valves can be stored indefinitely in liquid nitrogen, although the nature of any deterioration during storage is not well characterized. The viability of cryopreserved connective tissue matrix cells is maintained, but at a lower level than that of fresh valves,<sup>88</sup> and endothelial viability is lost.<sup>89</sup>

The aorta and iliac arteries can be preserved using the same methods applied to heart valves. Frequently, the aortic arch is preserved with the aortic valve intact; such grafts are intended for transplantation as a valved conduit. Preservation and storage methods are similar to those for valves. Synthetic grafts are the primary graft of choice, but such grafts may be less effective in an infected field. Aortoiliac arteries are used successfully as conduits in mycotic aneurysm repairs, when synthetic grafts have become infected, and for aortoenteric fistulas in an infected field.<sup>90</sup>

Arterial or venous segments of vascular organs may be recovered in order to provide a source of vascular "conduits" for use in organ transplants when the organ's attached vessels are damaged or inadequate. Vascular "conduits" that have been recovered and transplanted under these conditions are not considered tissues under the FDA's human cells, tissues, or cellular or tissue-based products (HCT/Ps) rules, but they are regulated as organs under 42 CFR Part 121. Donor screening and testing, as well as labeling and storage requirements, are identical to those for donor organs specified in a federal contract with the Organ Procurement and Transplantation Network.

Autograft veins are used in cardiac and peripheral vascular bypass graft procedures whenever it is possible, but veins from deceased donors that have been recovered under aseptic conditions may be used for revascularization when autologous mammary or saphenous vein grafts are not available. These veins are more fragile than are the great vessels, and they are more easily damaged during recovery and during thawing and preparation for implantation. Cryopreservation of veins is similar to that of cardiac allografts. Arterial grafts are seldom used for this purpose because their layers tend to separate during thawing. Well-established tissue bank procedures are designed to retain venous endothelial cells during recovery, processing, and preservation, but these cells are rapidly sloughed off the lumen after the vein is transplanted into the high-pressure arterial system. Retention of endothelial cells during recovery and processing does aid, however, in reduction of the risk of thrombosis or failure after implantation through protection of the integrity of the vessel's basement membrane.<sup>86</sup>

Although not proven to be necessary for successful clinical outcome or to prevent alloimmunization, ABO- and Rh-compatible allograft valves and vessel conduits are usually requested. Cryopreservation is known to alter the expression of antigens on donated cardiovascular tissue, but the mechanism of this effect is not well understood. Some studies have shown that the use of these tissue allografts may carry a risk of HLA antigen sensitization.<sup>91</sup>

## Peripheral Nerve

Fresh autografts of peripheral sensory nerves are used in nerve repair, but this practice is hampered by donor site morbidity and resulting limitations on the amount of autologous nerve tissue that can be made available. Although allografts ideally might repair peripheral nerve defects without requiring the sacrifice of the patient's own nerve, frozen, irradiated, and lyophilized allografts have not functioned well. New animal studies using nerve allografts cold-preserved for 7 weeks have shown promising results, as have cultured Schwann cells added into synthetic conduits.<sup>92</sup> Axogen, Inc. (Alachua, FL) has developed a thermally acellularized nerve allograft scaffold called Avance that is treated with chondroitinase in order to degrade chondroitin sulfate proteoglycan. Such grafts have been shown to inhibit both aberrant growth and retrograde regeneration in the absence of any immunosuppressive therapy. Animal studies employing such an approach demonstrated enhancement of nerve regeneration. The first human Avance nerve allograft was implanted in July 2007 into a 38-year-old man who had suffered a traumatic facial injury; a single nerve allograft was used to connect the severed nerve root to three nerve branches. The surgeons informally reported that the graft's handling characteristics were superior to those of autograft tissue, but it is too early to assess the extent and character of nerve regeneration.

## Parathyroid

Hypercalcemia, kidney stones, and other complications associated with hyperparathyroidism can be treated by surgical removal of the parathyroids. Hyperparathyroidism often is caused by a single parathyroid adenoma, but in 10% of cases, generalized parathyroid hyperplasia is found, rendering the removal of all four parathyroids necessary. Postoperatively, the lack of parathyroid hormone can result in permanent hypocalcemia in 5% of patients. To prevent this outcome, autotransplantation of a small amount of parathyroid tissue is performed during total parathyroidectomy in order to provide a controlled source of parathyroid hormone.<sup>93</sup> The parathyroid tissue is placed in the sternocleidomastoid muscle, flexor muscle groups, or subcutaneous tissue of the forearm. The remaining parathyroid tissue can be divided, placed in vials containing chilled tissue culture medium, and then cryopreserved using 10% autologous serum and 10% DMSO. The excess tissue then can be frozen under controlled conditions and stored in liquid nitrogen at  $-196^{\circ}\text{C}$ .<sup>94</sup> Frozen parathyroid autograft can be retrieved for subsequent use if the tissue implanted at time that the parathyroid was removed proves to be insufficient, fails to function, or becomes infected. Cryopreservation of parathyroid tissue with DMSO maintains cell viability and graft function. This is illustrated by postimplant amelioration of hypocalcemia and sustained elevation of parathyroid hormone in the venous effluent of the grafted forearm compared with that of the nongrafted forearm.<sup>95</sup> Postthaw viability can also be demonstrated in vitro by the suppression of parathyroid hormone secretion by the addition of increasing calcium concentrations.<sup>96</sup> The maximal duration of cryopreserved parathyroid tissue storage has not been determined.

## Reproductive Tissue

### Semen

Assisted reproductive technology procedures and artificial insemination of a female with her partner's previously stored semen or with donor semen are established therapies for the clinical management of infertility or when a woman does not have a male partner. Cryopreserved semen is used in both forms of artificial insemination. Cryopreserved semen can be stored by a man, termed a "client depositor," who may become sterile as a consequence of therapy for testicular malignancy or for another reason, for later use with his wife or other "intimate partner," or even with a surrogate mother. Sperm can even be collected postmortem, but such a practice poses ethical issues regarding the lack of consent. According to the FDA, there are approximately 110 semen banks in the United States. In 2005, semen banks licensed by New York State processed 27,118 ejaculates from 962 donors into 145,751 vials/straws in inventory. These banks distributed 74,366 vials/straws of semen for clinical use (New York State Department of Health, unpublished data, 2005).

Semen banks usually offer a library of donors from whom donated frozen semen specimens are available. The offering of a selection of donors facilitates the matching of donor's hair and eye color, race, and other genetically determined characteristics with those of the intended father or co-parent or with those of both parents. These donors are usually "anonymous"; such a donor's identity is known only to a few of the semen bank staff. However, some semen banks offer a program through which some donors have agreed to disclose their identities when offspring reach the age of 18 or, sometimes, even before use of the semen. Sexually transmitted diseases, including HIV infection, can be transmitted by donor semen to women undergoing artificial insemination.<sup>25,97,98</sup> Cryopreservation permits extended storage and the retesting of donors at least 6 months after the donation of specimens to be released. This process is intended to prevent use of semen donated by a recently infected man, before development of detectable antibody or viral nucleic acid (during the "window period"). Other diseases and organisms transmissible by donor semen include hepatitis B, gonorrhea, *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Trichomonas vaginalis*, and *Chlamydia trachomatis*. Transmission of HTLV-I, syphilis, HCV, and human papillomavirus may also be possible (see Table 58-4).<sup>25</sup>

The basic practices and techniques of semen cryopreservation have changed little since the cryopreservative glycerol was discovered accidentally by Polge and coworkers<sup>99</sup> in 1949. Glycerol remains the standard cryoprotectant, and liquid nitrogen remains in use for storage. Freezing methods in use have been designed to control the rate of temperature decline, and to prevent thermal shock initially by cooling the semen slowly in air or in a waterbath to  $5^{\circ}\text{C}$  before initiation of the actual freezing process, which takes place in the vapor phase of liquid nitrogen, or in a programmable controlled-rate freezing device. After freezing, semen can be stored in the liquid phase of liquid nitrogen indefinitely.

While a defective pregnancy as the result of sperm injury during the freezing-thawing process is a theoretic concern, such an effect has not been demonstrated. Cryopreservation of semen does not influence the frequency of abortions or multiple births, or the infant's gender, body size, or intelligence.<sup>100</sup> In fact, there is some evidence that indicates that the safety of cryopreserved semen actually might exceed that of fresh semen. One study reported finding birth defects in 0.7% of offspring and spontaneous abortion of 7.7% of pregnancies achieved using cryopreserved semen,<sup>101</sup> whereas in the general population the frequency of birth defects is 6% and the frequency of spontaneous abortion is 10% to 18% of pregnancies.

### Oocytes and Embryos

Since the birth of Louise Brown, the world's first "test tube baby," on July 25, 1978, there has been an explosion in the use of assisted reproductive technologies, such that several techniques have been accepted as standard medical therapy. There are at least 422 assisted reproductive technology programs in the United States.<sup>4</sup> While the technology to freeze unfertilized oocytes reliably is still in development, embryos are routinely cryopreserved. In 2005, >32,000 embryos, 13% of which were created using donor oocytes and/or donor semen, were transferred into patients in New York. In addition, >46,000 embryos were in storage in New York alone (New York State Department of Health, unpublished data, 2005). Many of those embryos were created using donor semen and/or donor oocytes. Donor oocytes were used in approximately 12% of all assisted reproductive technology cycles carried out in the United States in 2005.<sup>4</sup> Many of these were embryos created for recipients >35 years of age, using an oocyte donor who was much younger than the recipient. Embryo transfer success rates have been shown to be influenced far more by the age of the oocyte source than by the age of the uterus into which embryos are transferred. Medical history and infectious disease testing requirements similar to those for semen donors apply, although a quarantine period and retesting are not required.

### Extraembryonic Tissue Preservation and Transplantation

Extraembryonic tissues that have been used occasionally for transplantation include the amnion and the umbilical vein. Fetal amnion, which is the smooth, slippery, glistening membrane lining the fluid-filled space surrounding the fetus, has been used as a covering for nonhealing chronic leg ulcers, burns, and raw surfaces following mastectomy, and in major oral cavity reconstruction and vaginoplasty. Amnion also has been used as a pelvic peritoneum substitute following pelvic exenteration and as a source of replacement enzymes for infants with inborn errors of metabolism.<sup>102</sup> Most of the fetal amnion is covered on the maternal side by the chorion, a slightly roughened membrane. Amnion is sterilely collected during cesarean section. The amnion's epithelium and basement membrane can be separated by blunt dissection from the underlying chorion immediately after collection or after temporary storage. The amnion is then cryopreserved or lyophilized. Human umbilical vein allografts previously were used occasionally

as vascular substitutes to provide venous access for hemodialysis or as an arterial bypass graft, but such allografts proved to be inferior to saphenous vein autografts. Such umbilical vein grafts are no longer available, following application of FDA device manufacturing requirements to their recovery and processing.

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## Donor-Recipient Matching

For most tissues, donor-recipient HLA matching is not necessary and is rarely done. Tissues such as bone, fascia, tendon, cartilage, and dura mater are not preserved or transplanted in a viable state; rather, they serve as a support or matrix that the recipient's own cells can enter and gradually replace. Immunologic rejection, therefore, is not a significant concern, and matching of blood group or HLA antigens is considered unnecessary. There are exceptions, however. Immunologic rejection can occur in patients who have received a repeat cornea graft; therefore, efforts are made to use HLA-matched corneas in these patients.<sup>103</sup> HLA sensitization has also been reported in recipients of vascular allografts or allograft heart valves.<sup>104</sup>

The ABO antigens are a significant consideration in transplantation because they constitute very strong histocompatibility antigens. Because they are expressed on vascular endothelium, major ABO mismatching can cause rapid graft rejection resulting from endothelial damage by ABO antibodies and subsequent widespread thrombosis within the graft. Therefore, ABO matching is important to the success of vascularized organ grafts (ie, kidney, heart, liver, and pancreas). ABO matching is not important for a successful outcome when using most tissue grafts (ie, fascia, bone, heart valves, skin, and cornea). However, hypersensitivity to antigens expressed by fresh or cryopreserved donor tissue is a rare occurrence and appears to be dependent on an undetermined unusual immune response by the recipient.

Alloimmunization to Rh(D), Fy(a), and Jk(b) red cell antigens following transplantation of frozen unprocessed bone has been reported.<sup>61,62</sup> Consequently, frozen unprocessed bone allografts usually are matched with the donor for the D antigen if the recipient is a female of childbearing potential, in addition to being matched for ABO group.

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## Transfusion Service Support of Tissue Transplantation

Hospital transfusion services have been greatly affected by transplantation, and have encountered new and increased demands for services. They are involved in transplantation in several ways, including: 1) providing traditional blood components, 2) providing new or special blood components, 3) taking responsibility for tissue acquisition, storage, distribution, and tracking, and 4) providing specialized services. For organ transplants, the major demand is for traditional blood components, although special preparation may be required (see Chapter 40).

FDA regulations for tissue (see below) cover donor selection and testing, tissue recovery, processing, storage, labeling, and distribution to the “consignee” (21 CFR Part 1271). The consignee can be a distributor, a surgeon in a hospital operating room, a dentist in his or her office, or a designated individual or department in a hospital or other health-care institution. Tissue “banks” that are located in hospitals are not regulated by the FDA if they serve only to store and dispense tissues provided by comprehensive tissue banks or distributors. A hospital tissue service can be centralized in a support area for the operating rooms, hospital central supply services, or the hospital transfusion service. Alternatively, tissues can be handled using a decentralized system and be ordered, received, and stored by each functional area of the hospital in which they are used.

Currently, most human deceased donor tissue (bone, skin, heart valves, veins, and tendons) is recovered by an organ and tissue procurement agency or regional tissue bank, sent for graft processing and packaging, and then distributed directly to the operating room or to other hospital sections in which tissue transplantation occurs. However, in the absence of centralization, records of storage and recipient identification may be inadequate. In one case involving an HIV-infected donor, the recipients of six of the tissues could not be identified from hospital records.<sup>16</sup> The Joint Commission standard on record-keeping and traceability of tissues (PC 17.20), College of American Pathologists transfusion medicine checklist (TRM.45250, Phase II), and AABB *Standards*<sup>105</sup> (Standards 5.1.6.2, 5.19.6.1, and 6.2.3) require that the organization’s records permit tracing of any tissue from the donor or source facility to all recipients or other final tissue disposition. However, New York State is the only government regulatory agency that requires centralized tracking of tissues to the recipients.

The hospital transfusion service has the capacity, experience, and skills to act as a central depot and distribution point for all human tissue and to ensure that storage, issuance, and disposition records are maintained. Functions include allograft selection; vendor qualification and price negotiation; receipt of tissues, including inspection and accessioning; proper storage; inventory control; issuance; and record-keeping, which must ensure traceability to recipient or other final disposition.<sup>106</sup> Record-keeping, especially if barcodes are used in the laboratory, is complicated by the fact that barcodes are not yet standardized among tissue banks, although adoption of ISBT code 128 labeling standards has been suggested.<sup>107</sup> An additional challenge is that some tissues are produced in lots or batches, so each individual unit may not carry a unique identifier as is the case with blood components. Effective development of such tissue storage and distribution services takes time and relies on good relationships and communications with both operating room staff who will handle tissues and the surgeons who use them.<sup>106</sup>

A transfusion service operating as a central tissue repository and dispensing service may also be called upon to manage autologous tissues, such as calvaria (skull bone flaps), bone, skin, and parathyroid gland. Such tissues may require preparation and packaging before storage. Testing is not required, but careful

labeling and record-keeping are essential. Such tissues may ultimately be reimplanted in the original location (such as calvaria) or in a heterotopic location (such as limbs for parathyroid gland). It is prudent to establish time limits for storage either on an individual basis as specified by the surgeon or on a generalized basis, because stored tissues may not be claimed if the tissue was not needed because the patient died or for other reasons. The tissue dispensing service may also be called upon to package tissues in an appropriate, qualified, properly labeled transport container for transport to another institution.<sup>106</sup>

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## Reimbursement

Reimbursement for tissue transplantation is similar to that for blood transfusion. The tissue bank recovers expenses through a service fee (per tissue) billed to the hospital. This service fee includes such costs as services rendered by the organ/tissue recovery agency; recovery supplies and logistical support of the recovery agent that may be provided by the tissue processor; the tissue processor’s operating costs associated with processing, storage, and distribution, as well as research and development; and overhead costs incurred with support of all operations. Health-care insurance carriers reimburse hospitals for most tissue service fees. Current procedural terminology codes specific to allograft transplantation procedures are available and used routinely.

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## Oversight

With the rapid growth of all areas of tissue banking, there has been an increasing need for accountability and for measures that ensure that safe, quality tissues are available for clinical use. Quality improvement can be effected through voluntary standards, and most tissue banks have incorporated the achievement of high standards into their goals. The AATB has established comprehensive standards for donor screening, recovery and processing of musculoskeletal, cardiac, vascular, and skin tissues, and reproductive cells.<sup>10</sup> Additionally, the standards contain: institutional requirements; descriptions of required functional components of a tissue bank; requirements for construction and management of records and development of procedures; requirements for informed consent, tissue labeling, storage, and release; expectations for handling adverse outcomes, investigations, and tissue recalls; requirements for establishment of a quality program; specifications for equipment and facilities; and guidelines for tissue dispensing services and tissue distribution intermediaries. AATB’s *Standards for Tissue Banking* are referenced not only by tissue bankers, but also by end-user health-care facilities, other standard-setting organizations, and regulators worldwide. In 2008, 104 tissue banks in North America held AATB accreditation.

AABB *Standards for Blood Banks and Transfusion Services*<sup>105</sup> address tissue inspection (Standards 4.3 and 5.10), handling (Standard 5.1.5), storage (Standard 5.1.8), preparation and

dispensing (Standard 5.15.3), handling adverse events (Standard 7.4.4), and record-keeping, which must provide traceability to each recipient or other disposition (Standards 5.19.6.1, 5.1.6.2, and 6.2.3). The Joint Commission implemented standards for storage and issuance of tissue for hospitals, ambulatory surgery centers, effective July 1, 2005 (Standards PC.17.10, PC.17.20, PC.17.30). These standards apply to bone, tendon, fascia, and cartilage, as well as other cellular tissues of both human and animal (xenograft) origin. The standards address key functions, including the need to develop procedures for tissue acquisition and storage, record-keeping and tracking, and follow-up of adverse events and suspected allograft-caused infections, which must be reported to the tissue bank from which the tissue was obtained. Similar to federal regulations and AATB *Standards*, the minimal record retention period is specified to be 10 years from the date of transplantation, distribution, other disposition, or expiration, whichever is latest. The College of American Pathologists' laboratory accreditation program's transfusion medicine checklist includes several questions on storage and issuance of tissues, including accountability; procedures for proper storage, handling, in accordance with the source facility's directions; procedures for investigating recipient infections and adverse events, and handling look-back notifications from a supplier; and record-keeping, which allow for tracking from donor to recipient and vice versa (TRM.45050 - TRM.45250).

Until recently, large segments of the tissue banking industry have been unregulated by government agencies. A few states, specifically New York, Florida, and California, adopted licensure requirements in the early 1990s. The New York State Department of Health has the most comprehensive oversight program. Beginning in 1991, New York established administrative, licensure, and technical requirements for all entities that collect, process, store, and/or distribute tissue in New York. Currently, 817 tissue banks, including 79 banks that recover and/or process cardiovascular, musculoskeletal, and/or skin tissue from deceased donors; 18 eye banks; 64 semen banks; 39 oocyte-embryo banks; 63 hematopoietic progenitor cell banks; and 84 nontransplant anatomic banks (banks providing bodies, body parts, and tissues for health professional education and medical research), are licensed to operate in New York. New York State regulations extend to the clinical use of tissue, such as physicians' offices where semen is used in artificial insemination and hospitals and ambulatory surgery centers that transplant tissue. Such transplantation services constitute the remainder of licensed tissue banks. A 2001 federal report found, however, that 44% of tissue banks held neither accreditation by a professional organization nor licensure by a state agency.<sup>13</sup>

FDA authority to create and "enforce regulations necessary to prevent the introduction, transmission, or spread of communicable diseases between the States or from foreign countries into the States" under section 361(a) of the US Public Health Service Act (42 USC 264) applies to human tissue intended for transplantation. Formal enforcement policy and regulations did not exist until December 14, 1993 (codified in 21 CFR Parts 16

and 1270), when the "Interim Rule: Human Tissue Intended for Transplantation," which required donor screening, infectious disease testing and record-keeping "to prevent transmission of infectious diseases through human tissue used in transplantation," was adopted in response to reports of HIV transmission by human tissue and of potentially unsafe bone imported into the United States.<sup>108</sup> Reproductive tissue, human milk, and marrow were specifically excluded.

These regulations were supplanted by a series of federal regulations, published in stages, first announced in the Proposed Approach to the Regulation of Cellular and Tissue-Based Products in March 1997.<sup>109</sup> A final rule, "Human Cells, Tissues, and Cellular and Tissue-Based Products: Establishment Registration and Listing," published in January 2001, required organizations that are engaged in tissue recovery, donor qualification, tissue processing, and/or tissue-related laboratory testing to register as a tissue establishment with the FDA. The rule (21 CFR Part 1271) became effective for all tissue banks on March 29, 2004.

A final rule, "Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products," published May 25, 2004, set forth donor eligibility requirements, including health history screening and laboratory testing. Another final rule, "Current Good Tissue Practice for Human Cell, Tissue and Cellular and Tissue-Based Product Establishments; Inspection and Enforcement," published on November 24, 2004, established elements of good tissue practice, analogous to good manufacturing practice for blood banks. Both rules became effective May 25, 2005 (see 21 CFR Parts 1270 and 1271). An interim final rule, published on May 25, 2005 for immediate implementation, made a few technical corrections regarding donor screening and testing and product labeling. Following consideration of comments regarding the interim final rule, the final rule was published on June 19, 2007, but was unchanged from the May 25, 2005 rule (see 21 CFR Parts 1270 and 1271).

In addition to requirements for establishment registration, donor eligibility screening and testing, and good tissue practice, the regulations set forth requirements for adverse reaction reporting and also define inspection and recall authority. To improve tissue safety and surveillance, the FDA Current Good Tissue Practice Rule, effective May 25, 2005, requires that tissue establishments report infectious adverse events after allograft transplantation to the FDA through its MedWatch adverse event reporting system. In fiscal year 2006, the first full year of the requirement, 70 reports were filed by tissue banks; 44 of these reports were flagged to indicate possible recall of tissue(s). Of these flagged reports, the majority (26) pertained to acceptance of ineligible donors for whom one or more components of the donor qualification process was not performed or was insufficiently documented. The FDA also encourages health-care professionals, patients, and consumers to voluntarily report tissue adverse reactions through the MedWatch system (see <http://69.20.19.211/CBER/tissue/hctadverse.htm>). The CDC is currently funding efforts to develop the Transplantation Transmission Sentinel Network, which is intended to facilitate

recognition of adverse events associated with transplanted allografts. The system is being developed by UNOS, in collaboration with several stakeholders, including the AATB, EBAA, Association of Organ Procurement Organizations, American Academy of Orthopaedic Surgeons, American Orthopaedic Society for Sports Medicine, Society of Thoracic Surgeons, HRSA, and FDA.

## Acknowledgments

The authors thank Scott Brubaker, Lisa Eisenlohr, Patricia Dahl, and Perry Lange for their technical advice and Marcia Kolakowski for her assistance with manuscript preparation.

## Disclaimer

The authors have disclosed no conflicts of interest.

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Adoptive immunotherapy can be defined as the transfer of cellular products with the goal of increasing immunity against cancer or infections. A number of populations have been evaluated in clinical trials including donor leukocyte infusions (DLI) from allogeneic hematopoietic stem cell transplantation (HSCT) donors, activated T-cell population cells, natural killer (NK) cells, and antigen-specific T cells. At present, there is a defined role for adoptive immunotherapy using T-cell approaches in treating relapse of certain malignancies and some types of infection after allogeneic HSCT.<sup>1-5</sup> Extending this modality of treatment to patients with malignancies outside the HSCT setting is theoretically attractive, but successful applications are currently limited to settings where the tumors express immunogenic antigens such as viral antigens. This chapter reviews the current status of adoptive immunotherapy strategies for malignant and infectious diseases in the clinic and current areas of research to improve and extend the applicability of this approach.

### Targets for Immunotherapy for Cancer

A necessary prerequisite for T-cell immunotherapy approaches for cancer is identification of target antigens on the tumor cells. Advances in genomics have facilitated the identification of putative tumor antigens through the use of technologies such as serologic analysis of recombinant cDNA expression libraries and bioinformatics tools to deduce epitopes from candidate genes. However, for a tumor to be susceptible to immunotherapy approaches it must not only contain unique proteins to provide epitopes for targeting by specific immune responses, but must also present these peptides frequently enough and for sufficient duration to engage responder T cells. In addition, either the tumor cell or a specialized antigen-presenting cell through the

process of cross-priming must express major histocompatibility complex (MHC) antigens and express costimulatory molecules such as CD28 to induce T-cell activation.

Potential antigens for targeting tumor cells fall into several major categories (Table 59-1).

#### Alloantigens

In the setting of allogeneic HSCT, alloantigens that differ between donor and recipient are targets for T-cell recognition. Alloantigens include human MHC molecules (in HLA-mismatched transplants) and minor histocompatibility antigens, which are naturally processed peptides derived from normal cellular proteins where different polymorphisms are present in donor and recipient.<sup>18,19</sup> A number of minor histocompatibility antigens have been identified, and recent data showing that responses to donor lymphocyte infusions correlate with increase in immune responses directed against these antigens confirm their importance.<sup>20,21</sup> In some of these cases, alloreactivity also results in graft-vs-host disease (GVHD), but in cases where the minor antigen targeted is selectively expressed on hematopoietic cells, a graft-vs-leukemia (GVL) effect can occur in the absence of GVHD.

#### Virus-Associated Tumor Antigens

Some cancers (eg, subsets of lymphomas and nasopharyngeal carcinomas) are associated with viruses that present unique epitopes that are usually highly immunogenic as targets for a T-cell response. Latent Epstein-Barr virus (EBV) infection is associated with a heterogeneous group of malignant diseases (Fig 59-1) including posttransplant lymphoproliferative disease (PTLD), Hodgkin disease, nasopharyngeal carcinoma, and gastric carcinoma.<sup>4,22,23</sup> The role of EBV in tumorigenesis is well established in the EBV-LPDs that arise in immunosuppressed individuals but is less clear in other EBV-associated malignancies. Nevertheless, the presence of EBV in tumor cells offers a target for immunotherapy approaches. Human herpesvirus-8 (HHV-8) is primarily associated with Kaposi's sarcoma and primary effusion lymphoma in patients with AIDS, but is also found in some solid tumors.<sup>24</sup> Unlike most malignant cells, which

**Table 59-1.** Potential Targets for Immunotherapy of Cancer

| Target                  | Examples                                          | Malignancies                                                                                  |
|-------------------------|---------------------------------------------------|-----------------------------------------------------------------------------------------------|
| Viral antigens          | Epstein-Barr virus                                | Non-Hodgkin lymphoma <sup>6</sup><br>Hodgkin disease <sup>7</sup><br>Nasopharyngeal carcinoma |
|                         | SV40                                              | Non-Hodgkin lymphoma <sup>8</sup>                                                             |
| Differentiation antigen | CD19 or CD20                                      | Leukemias and lymphomas <sup>9</sup>                                                          |
|                         | CD30                                              | Hodgkin disease and non-Hodgkin lymphoma <sup>10</sup>                                        |
| Cancer testis antigens  | MAGE antigens<br>MART-1<br>NY-ESO<br>SSX antigens | Testicular, melanoma, many carcinomas, and many hematologic malignancies <sup>11-13</sup>     |
| Minor antigens          | HA1, HA2                                          | After transplantation for leukemia and lymphoma <sup>14</sup>                                 |
| Tumor-specific antigens | Telomerase                                        | Many types of malignancies <sup>15</sup>                                                      |
|                         | Idiotype                                          | Non-Hodgkin lymphoma <sup>16,17</sup>                                                         |

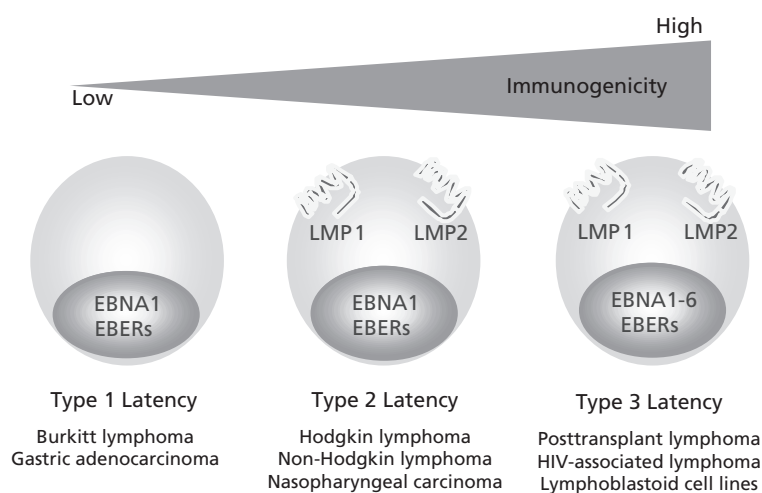


**Figure 59-1.** Type of Epstein-Barr-virus (EBV) latency. The three types of EBV latency are defined by the number and type of latent protein expressed on EBV-infected B cells and EBV-positive tumor cells.

probably express small quantities of a single modified peptide as a cytotoxic T lymphocyte (CTL) target, virus-transformed cells may express a range of viral antigens in high copy number. These cells are, therefore, potentially much more susceptible to T-lymphocyte-mediated immunotherapy than tumor cells transformed by other mechanisms.

### Lineage-Specific Antigens

Differentiation antigens selectively expressed in tumor cells provide another potential target. In the context of hematologic malignancies, antigens such as CD19 and CD20 (B-lineage), CD33 (myeloid), and CD30 (expressed on Hodgkin's Reed Sternberg cells) have been successfully targeted with antibody therapy, and preclinical studies have identified peptides that may be targets for T-cell recognition.<sup>25,26</sup>

### Universal Antigens

Several antigens have been identified that are overexpressed in a number of different tumor cells including telomerase and

cytochrome P450 1B1.<sup>15,27</sup> Antigens that are crucial for tumor cell function such as cyclin or other cell cycle genes are other potential targets.<sup>28</sup>

### Cancer Testis Antigens

The cancer testis antigens (CTAs) comprise, to date, 44 families of genes or isoforms. Among these, 19 families are purely testis-restricted, while others are expressed in one or more somatic tissues.<sup>11</sup> The physiologic expression of CTAs within the testis prevents their effective presentation to the immune system; hence, for those expressed only in testis, T-cell tolerance does not appear to be established. Expression of the MAGE-1 CTA was first shown in melanoma, but subsequently, expression of multiple CTAs was demonstrated in a variety of tumors.<sup>11-13</sup> The mechanisms underlying the expression of CTAs are only partially resolved. Epigenetic regulation seems critical and demethylation of CTA promoter sequences has been observed in malignant cells.<sup>11</sup> CTA-specific T cells able to destroy tumor cells were first identified in patients with melanoma, but subsequently were

isolated from patients with a range of tumors<sup>29,30</sup> and have the potential to be used as a target for many cancer immunotherapies.<sup>31,32</sup> For example, NY-ESO-1 is frequently expressed in melanoma and poor prognosis B-cell malignancies and is capable of eliciting spontaneous humoral and T-cell immunity.<sup>30</sup> However, it remains a challenge to expand CTLs specific for such tumor antigens in vitro to the numbers required for clinical use.

## Targets for T-Cell Immunotherapy for Infections

In the setting of immunotherapy for viral or fungal infection, it is also crucial to know which antigens are most important for viral/fungal persistence and thus the best targets for immunotherapy strategies. The viral antigens expressed at different stages of viral infection have been well characterized for some viruses such as cytomegalovirus (CMV) and more recently adenovirus, but are less well defined for other viruses that cause morbidity such as BK virus.<sup>33,34</sup> Most published studies designed to assess the immune response to fungal antigens (eg, *Aspergillus fumigatus*) use crude extracts from mycelia and spores. Numerous recombinant *A. fumigatus* allergens associated with allergic bronchopulmonary aspergillosis have been characterized but most elicit humoral immune responses and others are toxic to peripheral blood mononuclear cells (PBMCs), are strongly homologous to human proteins, or only prime a Th2 T cell response.<sup>35</sup> Therefore it is critical to identify allergens (eg, Asp f2 and Asp f16) that have been shown to stimulate both T- and B-cell responses from patients. Over the past few years, however, antigen identification has been simplified by the availability of tools that have enabled peptide mapping of specific viral and fungal epitopes recognized by CD4+ and CD8+ T cells.<sup>34,36,37</sup>

## Types of Adoptive Immunotherapy

Immunotherapeutic approaches that have been evaluated in the clinic can be broadly divided into studies using 1) nonspecific T cells, 2) antigen-specific T cells, 3) genetically modified T cells, and 4) NK cells.

### Nonspecific T Cells

#### Unmanipulated Allogeneic T Cells (Donor Lymphocyte Infusion)

Perhaps the most successful T-cell immunotherapy to date has been the use of unmanipulated allogeneic DLI following allogeneic HSCT. DLI has been most successful in patients with chronic myeloid leukemia who relapse after transplantation (70%-80% cytogenetic remission rate)<sup>5</sup> or with BBV-LPD (up to 90%).<sup>4,38</sup> Moderate success has been seen when DLI was used after relapse in other malignancies such as: acute myeloid leukemia (15%-40%), low-grade lymphomas (~60%), and metastatic multiple myeloma (40%-60%).<sup>39-42</sup> Less than 5% of patients with relapsed acute lymphoblastic leukemia (ALL) respond to

DLI alone. Although the etiology is unclear, this could be caused by lack of antigenic expression, downregulation of T-cell recognition molecules, and/or overall tumor burden at the time of treatment.

Although treatment with DLI can induce remissions in patients with disease after HSCT, unmanipulated cells also contain alloreactive T cells and can induce GVHD. The incidence of GVHD ranges from 55% to 90% and is associated with a 20% treatment-related mortality rate.<sup>5</sup> Nevertheless, studies evaluating chronic myelogenous leukemia (CML) responses to DLI have found that up to 55% of patients who do not experience GVHD may have a disease response,<sup>43,44</sup> demonstrating that GVL effect can be separated from GVHD. Therefore, one strategy to reduce the risk of GVHD is to infuse escalating doses of T cells in an attempt to obtain the GVL effect without GVHD. Peggs et al<sup>45</sup> conducted a dose escalation study using DLI after HSCT for patients with hematologic malignancies and showed that in sibling transplantation, higher DLI doses were associated with a significantly increased risk of GVHD. In addition GVHD was more common, occurred at lower T-cell doses, and was more severe in the unrelated donor cohort, likely reflecting the increased alloreactivity between donor and recipient.

As another approach to separate GVHD and GVL effect with DLI, investigators have evaluated specific subsets of lymphocytes, such as CD4-selected cells,<sup>46</sup> CD8-depleted cells,<sup>47</sup> or functionally defined subsets such as Th2 cells.<sup>48</sup> In animal models, the culture of functionally defined T-cell subsets, such as Tc2 or Th2 cells, has allowed antitumor effects to be produced in the absence of alloreactivity. A recent Phase I clinical trial<sup>49</sup> at the National Institute of Health (NIH) evaluated the feasibility of infusing donor CD4 Th2 cells generated ex vivo using CD3/CD28 costimulation in the presence of interleukin (IL)-4 and IL-2. The patients had hematologic malignancies and underwent reduced-intensity conditioning regimens before HSCT. The cells were from a matched sibling donor who had undergone granulocyte colony-stimulating factor (G-CSF) mobilization. Nineteen patients received no additional cells, and the remaining 28 received these Th2-like cells in a dose-escalation fashion (5, 25, or 125 × 10<sup>6</sup> cells/kg). The Th2 cohort had accelerated lymphocyte reconstitution with acute GVHD and overall survival similar in both the Th2 and non-Th2 cohorts.<sup>49</sup>

### Allodepleted T Cells

Another approach to overcome the problem of alloreactivity is to selectively deplete the T-cell product of alloreactive cells expressing activation markers in response to alloantigen. Alloreactive cells express activation markers after exposure to alloantigen, including CD69, CD147, and the IL-2 receptor CD25, which is expressed within the first 24 hours of T-cell activation. This approach has been evaluated in three clinical trials using a CD25 immunotoxin to selectively deplete alloreactivity, two involving haploidentical CD34 selected grafts in predominantly pediatric patients and one involving HLA-identical sibling transplants in adults.<sup>50-52</sup> In all studies the approach was feasible and safe

and was able to promote immune reconstitution and recovery of virus-specific immunity without significant GVHD. Further studies to evaluate if there is an antitumor effect are continuing.

### Regulatory T Cells

An alternative option to prevent GvHD is to actively downregulate alloreactive T cells by recruiting regulatory T cells (Treg cells). Treg cells suppress the proliferation of CD4+ and CD8+ T cells after polyclonal or antigenic stimulation in a cell contact-dependent and cytokine-independent manner.<sup>53</sup> If CD4+, CD25+, FoxP3+, Treg cells, which are naturally present in the transplant product, are depleted before infusion into irradiated allogeneic recipient mice, then GVHD is significantly accelerated. Conversely, GVHD can be prevented by the addition of freshly isolated Treg cells to the donor product and of note the GVL effect is spared.<sup>54</sup> Supporting the importance of this effect, studies in humans have shown that a higher frequency of Treg cells in donor cells correlates with a lower risk for developing GVHD. Several groups are now investigating the value of infusing ex-vivo expanded donor Treg cells following HSCT. Many obstacles to the effective implementation of this approach remain, such as the difficulty of using cell sorting to obtain a pure Treg-cell population for infusion, given that FoxP3, the best Treg-cell marker to date, is intracellular.

Ongoing clinical trials using Tregs include the transfer of IL-10-aneurized  $T_H1$  cells to prevent GVHD in patients after HLA-haploidentical HSCT. In one trial,<sup>48</sup> donor PBMCs are thawed and cultured ex vivo in the presence of irradiated host PBMCs (obtained before HSCT) and IL-10. These IL-10-aneurized donor T cells are then infused at increasing doses starting from  $10^5$  per kg.<sup>55</sup> The first clinical trial with Treg cells is ongoing in Regensburg, Germany.<sup>56,57</sup> In this study, cancer patients with a high risk of relapse after allogeneic HSCT receive a preemptive DLI after stopping immune suppressive therapy and then 8 to 10 weeks later receive between  $1 \times 10^6$  and  $5 \times 10^6$  freshly isolated donor CD4+/CD25+ Treg cells/ kg of body weight followed by DLI of equal T-cell numbers. In the first five patients sufficient numbers of donor Treg cells were obtained and no toxicities (eg, GVHD, infection, and relapse) were seen.<sup>55</sup> This trial and others that are planned will provide critical information regarding the ex-vivo expansion of Treg cells and on the feasibility and safety using unmanipulated Treg cells for adoptive immunotherapy.

### Autologous Nonspecifically Expanded T Cells

Over the past few years several studies have correlated faster recovery of lymphocyte counts with improved outcome after autologous transplant for some malignancies.<sup>58,59</sup> To determine if T-cell recovery after autografting can be accelerated, June and colleagues have administered T cells expanded ex vivo using anti-CD3 and anti-CD28 to patients with myeloma and non-Hodgkin lymphoma (NHL) after autografting and showed rapid reconstitution of lymphocytes.<sup>60,61</sup> In the myeloma study,<sup>61</sup> immunotherapy was combined with vaccination and a single, early, posttransplant infusion of in-vivo vaccine-primed and

ex-vivo-costimulated autologous T cells. This treatment was followed by posttransplant booster immunizations, which led to the rapid induction of clinically relevant immunity as well as improved T-cell proliferation in response to antigens that were not contained in the vaccine.<sup>61</sup> These results show that adoptive transfer of autologous CD3/CD28-stimulated T cells is feasible and safe and that the combination of vaccine therapy and adoptive T-cell transfer leads to enhanced memory T-cell responses. Follow-up studies are evaluating effects on tumor responses.<sup>62</sup>

Another autologous ex-vivo expanded immunotherapy product that has been evaluated in the clinic is cytokine-induced killer (CIK) cells, a unique population of CTLs with a characteristic CD3+ CD56+ phenotype. In a clinical study, patients with relapsed NHL or Hodgkin disease were treated with escalating doses of autologous CIK cells generated by culturing apheresis products with interferon- $\gamma$ , OKT3, and IL-2.<sup>63</sup> No significant toxicity was seen and there were partial responses and disease stabilizations.<sup>63</sup> Because these were heavily pretreated patients who had all relapsed after transplantation, this approach may have greater activity in a setting of minimal residual disease.

### Antigen-Specific T-Cell Therapies

The use of antigen-specific CTLs may overcome the problem of alloreactivity and may also allow expansion of low-frequency cells. To generate these cells ex vivo requires a defined antigen and an antigen-presenting cell that can effectively present the antigen to T cells with appropriate costimulatory signals. Therefore, it is a significant challenge to generate T cells specific for tumor antigens if the malignant cell presents antigen poorly, and the putative target antigens are weak. However, proof of principle has been shown by studies administering donor-derived CTLs specific for viral antigens to reconstitute immunity following HSCT.

### EBV-Specific Cytotoxic T Cells

Immunotherapeutic strategies aimed at targeting EBV have now been used for over 15 years. All EBV-associated malignancies are associated with the virus' latent cycle, and three distinct types of EBV latency have been characterized<sup>64,65</sup> with varying expression of EBV latent proteins (Fig 59-1). Latency Type III is expressed in lymphoblastoid cell lines (LCLs), which can be readily produced by infecting B cells in vitro with EBV and is characterized by expression of the entire array of nine EBV latency proteins. This pattern of EBV gene expression characterizes the EBV-LPD that occur in individuals severely immunocompromised by solid-organ or stem cell transplantation, congenital immunodeficiency or human immunodeficiency virus (HIV) infection. Latency Type II is the hallmark of EBV-positive Hodgkin disease where a more restricted array of proteins including EBNA1, BARF0, LMP1, and LMP2 are expressed. In latency Type I, found in EBV-positive Burkitt lymphoma only EBNA1 and BARF0 are expressed. Because EBNA1 is not well processed by the Class I processing machinery,<sup>66</sup> tumor cells expressing Type I latency may not be a good target for immunotherapy approaches. However,

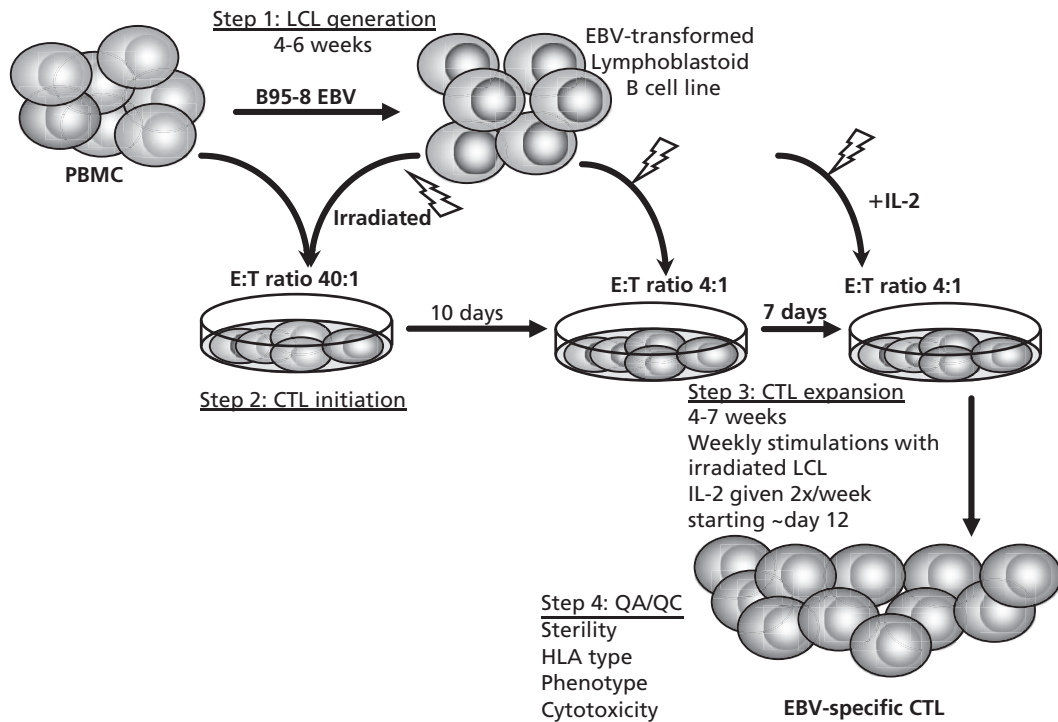


**Figure 59-2.** Generation of Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes.

Step 1: Peripheral blood mononuclear cells (PBMCs) are infected with a laboratory strain of EBV (B95.8) to generate an EBV-transformed B lymphoblastoid cell line (LCL).

Step 2: Irradiated LCLs are antigen-presenting cells to stimulate T cells specific for EBV at an effector to target (E:T) ratio of 40:1.

Step 3: The EBV-specific cytotoxic T lymphocytes (CTLs) are stimulated with irradiated LCL at weekly intervals at an E:T ratio of 4:1 starting from Day 10. Interleukin (IL)-2 at 50-100 U/mL is added twice weekly to the cultures from Day 12. The stimulations with LCL and IL-2 is continued until sufficient numbers of T cells have been expanded.

Step 4: CTLs are frozen and undergo extensive quality assurance/quality control (QA/QC) including HLA typing, phenotyping, assessment of HLA type, phenotype, and cytolytic function before being released for clinical use.

immunotherapy approaches targeting EBV antigens do have potential for treating Type II and Type III latency EBV tumors.

#### **EBV-Associated Lymphoma after Transplantation**

Risk factors for development of EBV-PTLD include the degree of mismatch between donor and recipient, manipulation of the graft to deplete T cells, and the degree of immunosuppression used to prevent GVHD.<sup>4</sup> EBV-associated PTLD arising after marrow transplantation is an ideal model to evaluate the ability of T cells to target EBV-positive tumor cells because these tumors are highly immunogenic expressing all nine latent cycle EBV antigens, including the immunodominant EBNA3 antigens. Furthermore, the outgrowing EBV-infected B-cell tumors have the same phenotype and pattern of EBV gene expression as do LCLs, which are an excellent antigen-presenting cell (Fig 59-1).

EBV-specific T cells can be generated in vitro using donor lymphocytes infected with a laboratory strain of EBV to initiate an LCL line that is an excellent antigen-presenting cell for presenting viral antigens. Irradiated LCLs are used to stimulate PBMCs and expand EBV-specific CTLs (Fig 59-2). EBV-specific T-cell lines have been used as prophylaxis for EBV-induced lymphoma in over 60 patients after HSCT who underwent a

T-cell-depleted HSCT or who were transplanted for an EBV-associated malignancy<sup>4,6,67</sup> and none of the patients treated with this approach developed PTLD, compared with an incidence of 11.5% in a historical nontreated control group.<sup>6</sup> These results have been confirmed by another group.<sup>68</sup> (See Table 59-2.)

Immunotherapy using EBV-CTL has also been used to treat patients with overt lymphoma. This strategy was successful in five of six patients and showed by gene marking that CTL accumulated at sites of disease.<sup>6</sup> Other groups have also obtained encouraging response rates<sup>38,72</sup> including one study in patients who had recurrent PTLD after initial therapy with rituximab.<sup>75</sup> In another study, one patient who received CTL as treatment died with progressive disease 24 days after infusion.<sup>76</sup> In this patient, the cytolytic activity of the generated CTL line was directed mainly against two HLA-11-restricted epitopes in the EBNA3b gene. However, after CTL infusion only virus with an EBNA3b deletion could be detected, allowing the tumor to evade the immune response.<sup>76</sup> This experience suggests that it is preferable to infuse a product with broad specificity.

In recipients of solid-organ transplants, the severe impairment of T-cell function as result of the required immunosuppressive regimen after transplantation also places these patients at risk

**Table 59-2.** Clinical Trials on Use of EBV-Cytotoxic T Cells as Prophylaxis or Treatment for Posttransplant Lymphoproliferative Disease (PTLD) after Marrow Transplantation

| Study                                                       | No. of Patients                                            | Type of Transplant                                                                                                                      | Pathologic Evidence of PTLD                                     | Cytotoxic T-cell (CTL) Lines                                                                  | Results                                                                                                                                  |
|-------------------------------------------------------------|------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------|-----------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------|
| Rooney et al, <sup>6</sup><br>Heslop <sup>69</sup>          | 54                                                         | T-cell-depleted HSCT (mismatch related donor or matched unrelated donor)                                                                | No—prophylaxis study                                            | Allogeneic (donor-derived) EBV-CTL                                                            | No patients developed PTLD compared with 11.5% controls                                                                                  |
| Rooney et al, <sup>6</sup><br>Gottschalk et al <sup>4</sup> | 6                                                          | T-cell-depleted HSCT                                                                                                                    | Yes                                                             | Allogeneic (donor-derived) EBV-CTL                                                            | 5 CR, 1 died (no response to CTL secondary to tumor mutation resistant to CTL)                                                           |
| Gustafsson et al <sup>68</sup>                              | 6                                                          | T-cell-depleted HSCT or unmanipulated HSCT with ATG/ OKT3 conditioning (mismatched or matched unrelated donor or matched related donor) | No—Treatment based on ↑ EBV DNA levels                          | Allogeneic (donor-derived) EBV-CTL                                                            | 5 patients had ↓ EBV DNA levels; 1 patient subsequently died of PTLD (CTL showed poor specificity for EBV targets on cytotoxicity assay) |
| Lucas et al <sup>70</sup>                                   | 1                                                          | T-cell-depleted HSCT                                                                                                                    | Yes                                                             | Allogeneic (donor-derived) EBV-CTL                                                            | Patient attained CR                                                                                                                      |
| Imashuki <sup>71</sup>                                      | 1                                                          | Mismatched HSCT with ATG                                                                                                                | Yes                                                             | Allogeneic (donor-derived) EBV-CTL                                                            | Patient failed to respond                                                                                                                |
| Slobod et al <sup>72</sup>                                  | 40                                                         | T-cell-depleted HSCT (mismatch related donor or matched unrelated donor)                                                                | No—prophylaxis study                                            | Allogeneic (donor-derived) EBV-CTL                                                            | No patients developed PTLD                                                                                                               |
| O'Reilly et al <sup>38</sup>                                | 17 (includes 3 patients after solid-organ transplantation) | T-cell-depleted HSCT (mismatch related donor or matched unrelated donor)                                                                | Yes                                                             | Allogeneic (donor-derived) EBV-CTL                                                            | 11 CR, 2 disease stabilization                                                                                                           |
| Haque et al <sup>73</sup>                                   | 2                                                          | Allogeneic HSCT (unmanipulated graft)                                                                                                   | Yes                                                             | Closely matched allogeneic EBV specific CTL                                                   | 2 CR                                                                                                                                     |
| Leen et al <sup>74</sup>                                    | 11                                                         | Allogeneic HSCT (unmanipulated and T-cell- depleted grafts)                                                                             | Yes (n = 1)<br>Remaining patients had normal or high virus load | Multivirus specific polyclonal CD4+ and CD8+ T cells specific for CMVpp65, EBV and adenovirus | Patient with PTLD had CR with CTL alone<br>All cleared EBV with CTL alone                                                                |

EBV = Epstein-Barr virus; HSCT = hematopoietic stem cell transplantation; CR = complete response; ATG = antithymocyte globulin.

for the development of PTLD with incidence dependent of the type of organ transplanted and the degree of immunosuppression. Because reducing immunosuppression can often control LPD, administration of EBV-specific CTLs might be an alternate means of restoring the immune response. In this clinical scenario, the organ donor is not HLA-matched and often is not available, and the tumor often arises from recipient cells. Therefore, most studies in solid-organ recipients have used autologous CTLs generated from peripheral blood of transplant recipients.<sup>77-79</sup> The results of these studies are summarized in Table 59-3. These studies have shown that the approach is safe and that EBV-specific immunity can be transiently restored. However, the persistence of CTLs is less than that observed after HSCT transplantation, perhaps because of the continuing immunosuppression that these patients receive to prevent rejection.

An alternative approach is to use closely matched allogeneic EBV-specific CTLs. A Phase II clinical trial has evaluated this

strategy in HSCT and solid-organ recipients who had failed to respond to conventional PTLD therapy. Response rates were 64% and 52% at 5 weeks and 6 months, respectively.<sup>73</sup> Patients with closer HLA matching donors showed better responses at 6 months.

### **EBV-Associated Type II Latency Tumors**

EBV-associated nasopharyngeal carcinoma, Hodgkin disease, and NHL that develop in the immune competent host show Type II latency where viral gene expression is limited to the immunosubdominant latent membrane protein (LMP)1 and LMP2, EBNA1, and EBERs,<sup>85</sup> which are weak targets for CTL activity. Nevertheless, these subdominant EBV antigens are potential targets for immunotherapy approaches. Phase I dose-escalation studies have evaluated the use of autologous EBV-specific CTL for patients with EBV-positive Hodgkin disease or nasopharyngeal carcinoma.<sup>7,86</sup> The Hodgkin disease study involved



## Section V

**Table 59-3.** Clinical Trials on Use of EBV-Cytotoxic T Cells as Prophylaxis or Treatment for Posttransplant Lymphoproliferative Disease (PTLD) after Solid-Organ Transplantation

| Study                        | No. of Patients | Types of Solid-Organ Transplants Included | Pathologic Evidence of PTLD        | Cytotoxic T-cell (CTL) Lines                | Results                                                                 |
|------------------------------|-----------------|-------------------------------------------|------------------------------------|---------------------------------------------|-------------------------------------------------------------------------|
| Comoli et al <sup>78</sup>   | 7               | Heart, liver, kidney                      | Prophylaxis                        | Autologous EBV specific CTL                 | No patient developed PTLD                                               |
| Haque et al <sup>80</sup>    | 3               | Liver, kidney                             | Prophylaxis                        | Autologous EBV specific CTL                 | No patient developed PTLD                                               |
| Khanna et al <sup>77</sup>   | 1               | Lung                                      | Yes                                | Autologous EBV specific CTL                 | Significant regression                                                  |
| Sherritt et al <sup>81</sup> | 1               | Heart                                     | Yes                                | Autologous EBV specific CTL                 | CR                                                                      |
| Comoli et al <sup>82</sup>   | 5               | Kidney                                    | Yes                                | Autologous EBV specific CTL                 | CR (used as adjuvant after chemotherapy rituximab)                      |
| Sun et al <sup>83</sup>      | 2               | Kidney, liver                             | Yes                                | Closely matched allogeneic EBV specific CTL | 2 CR                                                                    |
| Haque et al <sup>84</sup>    | 7               | Liver, small bowel, kidney                | Yes                                | Closely matched allogeneic EBV specific CTL | 3 CR; 1 PR; 3 did not respond                                           |
| Savoldo et al <sup>79</sup>  | 12              | Liver, heart                              | Prophylaxis (n = 10) and treatment | Autologous EBV CTL                          | No patient developed PTLD after CTL; 1 attained CR; >1 PR (> 12 months) |
| Haque et al <sup>73</sup>    | 31              | Heart, liver, kidney, lung, small bowel   | Yes                                | Closely matched allogeneic EBV specific CTL | At 6 months after CTL: 12 CR, 3 PR, and 16 persisting/ progressive PTLD |

EBV = Epstein-Barr virus; CR = complete response; PR = partial response.

14 treated patients, of whom seven received gene-marked CTLs. The infused CTLs expanded *in vivo*, persisted up to 12 months and trafficked to tumor sites.<sup>7</sup> EBV-CTLs were well tolerated, and had some antitumor activity with five patients (two treated with active disease) remaining in complete remission up to 40 months, one having a partial response, and five having stable disease. Lucas and colleagues also saw activity using EBV-CTL from partially HLA-matched normal donors.<sup>87</sup> Five of six patients with Hodgkin disease had reductions in measurable disease and the maximal duration of response was 22 months.<sup>87</sup>

EBV-CTLs have been administered to 10 patients with EBV-positive nasopharyngeal carcinoma who were in relapse or who had high-risk disease.<sup>86</sup> The four patients with high risk disease remain in remission after treatment and two of the six patients in relapse entered complete remission and remained disease free 24 to 42 months after CTL infusion.<sup>86</sup> Comoli et al<sup>88</sup> also treated 10 patients with relapsed disease and saw responses in six—two with partial responses and four with stable disease.

Although these results using EBV-specific CTL in Type II latency tumors have shown some activity (Table 59-4), the response rate is less impressive than that seen in EBV-LPD after HSCT. Moreover, apart from the patients with relatively low tumor burden who have demonstrated durable responses, the majority of the antitumor responses have been transient, and no patient with aggressive, bulky relapsed disease has been cured by CTL therapy alone. This may be due to a lack of specificity of the EBV-specific CTLs for the immunosubdominant LMP1 and LMP2 antigens present on the Type II latency tumor (Fig 59-1).

A follow-up study has shown that LMP2-CTLs could be generated from normal donors using dendritic cells genetically modified with a recombinant adenovirus encoding

LMP2 (Ad5LMP2). for the initial stimulation, followed by stimulation with LCL that had been genetically modified to overexpress LMP2a or LMP1 and LMP2 by transduction with an Ad5f35LMP2a vector or a Ad5f35LMP1-I-LMP2 vector, respectively.<sup>91</sup> LMP2-specific CTL lines using this methodology were administered to 16 patients with EBV+ve Hodgkin disease or EBV-positive B-cell or T/NK-cell NHL.<sup>89</sup> The LMP2-specific CTLs expanded and persisted *in vivo* without adverse effects. Nine of 10 patients treated in remission of high-risk disease remain in remission, and four of six patients with active relapsed disease had a complete response.<sup>89</sup> These studies are being extended using autologous T cells enriched for both LMP1 and LMP2 for the treatment of EBV-positive lymphoma and nasopharyngeal carcinoma.

### CMV-Specific Cytotoxic T Cells

Inability to control CMV reactivation following allogeneic HSCT is caused not only by low antigen-specific T-cell numbers but also by impairment of the T-cell function *in vivo*.<sup>92</sup> CMV prophylaxis and treatment with adoptively transferred, donor-derived CMV-specific CTLs has been successfully used with CMV-specific CD8+ clones or polyclonal CTL lines (Table 59-5). In the first study from Riddell and colleagues, CMV-specific T-cell clones were derived from sibling donors after stimulation with autologous fibroblasts pulsed with CMV.<sup>2,99</sup> There were no adverse effects and CMV-specific immune responses were reconstituted, although long-term persistence of infused CTLs was seen only in recipients who developed recovery of CD4+ CMV-specific T-cell responses. Other groups have also reported successful reconstitution of immunity to CMV by administering CMV-specific CTLs generated by stimulation of PBMCs with

**Table 59-4.** Clinical Trials on Use of EBV-Cytotoxic T Cells as Treatment for Type II Latency EBV-Positive Tumors

| Study                         | Number of Patients | Type of Disease                                                                                                  | Cytotoxic T-Cell (CTL) Lines and Dose                                                                        | Results                                                 |
|-------------------------------|--------------------|------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------|---------------------------------------------------------|
| Sun et al <sup>83</sup>       | 4                  | EBV-positive HD and NHL (including 1 HIV associated and 2 after solid-organ transplant)                          | Allogeneic (2 partially HLA-matched and 2 from HLA matched siblings) EBV-CTL<br>$5 \times 10^6/\text{kg}$    | 1 CR, 1 CRU, 1 PR and 1 NR                              |
| Lucas et al <sup>87</sup>     | 6                  | Relapsed EBV-positive HD                                                                                         | Allogeneic (partially HLA matched) EBV-CTL.<br>$1.5 \times 10^7/\text{kg} \pm$ pretreatment with fludarabine | 1 CR, 4 PR, 1 NR (although ?effect of fludarabine)      |
| Bollard et al <sup>7</sup>    | 14                 | Relapsed EBV-positive HD—either as adjuvant treatment after HSCT or for treatment of active disease              | Autologous EBV-CTL<br>$4 \times 10^7/\text{m}^2 - 1.2 \times 10^8/\text{m}^2$                                | 5 CR (includes 2 with active disease), 1 PR, 5 SD, 3 NR |
| Bollard et al <sup>89</sup>   | 16                 | Relapsed EBV-positive HD or NHL—either as adjuvant treatment after HSCT/chemo or for treatment of active disease | Autologous LMP2-CTL<br>$4 \times 10^7/\text{m}^2 - 3 \times 10^8/\text{m}^2$                                 | 13 CR (includes 4 with active disease), 1 PR, 2 NR      |
| Comoli et al <sup>90</sup>    | 1                  | NPC—active disease                                                                                               | HLA matched (sibling) EBV-CTL                                                                                | SD (transient)                                          |
| Comoli et al <sup>88</sup>    | 10                 | NPC—all with active disease                                                                                      | Autologous EBV-CTL                                                                                           | 2 PR (3-5 months) and 4 SD (4-15 months)                |
| Straathof et al <sup>86</sup> | 10                 | NPC—either as adjuvant treatment after HSCT/chemo or for treatment of active disease                             | Autologous EBV-CTL<br>$4 \times 10^7/\text{m}^2 - 3 \times 10^8/\text{m}^2$                                  | 6 CR (includes 2 with active disease) 1 PR, 1 SD, 2 NR  |

EBV = Epstein-Barr virus; HD = Hodgkin disease; NHL = non-Hodgkin disease; HIV = human immunodeficiency virus; CR = complete response; CRU = complete response unconfirmed; PR = partial response; NR = no response; SD = stable disease; HSCT = hematopoietic stem cell transplantation; NPC = nasopharyngeal carcinoma.

dendritic cells pulsed with CMV antigens<sup>100</sup> or the HLA-A2-restricted peptide NLV derived from the CMV-pp65 protein.<sup>101</sup> CMV-specific CTLs have also been used therapeutically with encouraging results.<sup>93</sup> These methodologies for generating CMV-specific CTLs all require prolonged ex-vivo activation and expansion. To overcome this problem a recent study used tetramer selection of CMV peptide-specific T cells directly from peripheral blood and relied on expansion in vivo after adoptive transfer. Infused CD8+ T cells expanded by several logs after infusion, and cleared CMV infection in eight of nine cases.<sup>97</sup>

### Adenovirus-Specific T Cells

Another alternative to expand virus-specific T cells is to stimulate T cells with the infectious antigen of interest and then “capture” the responding cell that is producing effector cytokines such as interferon- $\gamma$ . This strategy has been used to isolate adenovirus-specific CTLs for clinical use. Cells were incubated with adenovirus lysate in vitro to produce CD4+ and CD8+ T cells that were highly specific for adenovirus and exhibited markedly reduced alloreactivity. Feuchtinger et al<sup>102</sup> infused adenovirus-specific CTLs generated using this approach into nine pediatric patients with systemic adenovirus infection following allogeneic HSCT. All patients tolerated the infusions, and they exhibited a dose-dependent and sustained in-vivo expansion of adenovirus-specific CTLs associated with clearance or decrease in viral load in five of six evaluable patients.<sup>102</sup> Only one patient developed acute exacerbation of chronic GVHD. The efficacy was independent of T-cell dose, implying in-vivo expansion in the presence of active viremia from even low numbers of transferred cells.

### Trivirus-Specific CTLs

Because it would be preferable to target more than one virus, methods have been developed to generate multivirus-specific CTLs for adoptive immunotherapy. The initial focus was on CMV, EBV, and adenovirus as common viral reactivations seen in the posttransplant period. Trivirus-specific T cells were reactivated by using mononuclear cells transduced with a recombinant adenoviral vector encoding the CMV antigen pp65 for the first stimulation followed by subsequent stimulations with EBV-LCLs transduced with the same vector. The resulting CTLs had specificity for all three viruses and were administered to 10 HSCT recipients.<sup>74</sup> After adoptive transfer there was recovery of immunity to CMV and EBV in all patients, but adenovirus-specific T cells were detected only in recipients who had evidence of adenovirus infection pre-infusion. Reactivations or infections with all three viruses were cleared. This approach is being extended to additional pathogens.

### Fungal-Specific T Cells

Although profound neutropenia following HSCT remains an important risk factor for opportunistic invasive fungal infections, most cases arise after neutrophil recovery in the context of potent immunosuppressive therapy for GVHD prevention or treatment. Perruccio et al recently detailed the results of the first use of *Aspergillus*-specific adoptive therapy following HSCT.<sup>103</sup> Ten patients with *Aspergillus* pneumonia and positive galactomannan antigenemia were treated with *Aspergillus*- and CMV-specific donor-derived T-cell clones. In all, galactomannan antigenemia decreased to normal within 6 weeks, with nine patients clearing the *Aspergillus* infection.<sup>103</sup>

**Table 59-5.** Published Reports on the use of CMV-Cytotoxic T Cells as Prophylaxis or Treatment for CMV Infection after Marrow Transplantation

| Study                         | No. of Patients | Type of Transplant                                               | Clinical Evidence of CMV Disease                            | Cytotoxic T Lymphocyte (CTL) lines                                                                        | Results                                                                                                                                    |
|-------------------------------|-----------------|------------------------------------------------------------------|-------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------|
| Walter et al <sup>2</sup>     | 14              | MRD marrow transplantation (unmanipulated graft)                 | No                                                          | CD8+ CMV-specific CTL clones                                                                              | Functional immunity detected in all; no patients developed CMV viremia or disease                                                          |
| Einsele et al <sup>93</sup>   | 8 (7 evaluable) | MRD, MMRD and MUD (4 patients received CD34 selected stem cells) | CMV colitis (n = 1)<br>CMV interstitial pneumonitis (n = 1) | Polyclonal CD4+<br>CD8+ CMV-specific CTL                                                                  | CMV DNA undetectable in 6/7 patients after CTL.<br>↑ functional immunity in 5 patients<br>2 nonresponders had increased immune suppression |
| Peggs et al <sup>94,95</sup>  | 16              | Allogeneic MRD or MUD                                            | No — administered when CMV DNA detected                     | Polyclonal CD8+ and CD4+ lines generated using CMV-lysate-pulsed antigen-presenting cells                 | Expansion of CMV-specific immune response<br>8 cleared virus with CTL alone<br>8 required antiviral agents                                 |
| Rauser et al <sup>96</sup>    | 8               | Allogeneic HSCT (unmanipulated graft)                            | No — administered when CMV DNA detected                     | Polyclonal CD8+ and CD4+ lines generated using CMV lysate and CMV-peptide-pulsed antigen-presenting cells | 6/7 cleared virus with CTL<br>1 patient did not respond                                                                                    |
| Cobbold et al <sup>97</sup>   | 9               | Allogeneic HSCT (unmanipulated graft)                            | No — administered when CMV DNA detected                     | HLA-peptide tetramer selected CD8+ T cells specific for pp65 and IE1                                      | 6 cleared virus with CTL alone<br>3 required antiviral therapy as well as CTL                                                              |
| Leen et al <sup>74</sup>      | 10              | Allogeneic HSCT (unmanipulated and T-cell-depleted grafts)       | No                                                          | Multivirus specific polyclonal CD4+ and CD8+ T cells specific for CMVpp65, EBV, and adenovirus            | Functional CMV immunity detected in all<br>None required antiviral agents<br>All cleared virus with CTL alone                              |
| Mackinnon et al <sup>98</sup> | 7               | Allogeneic HSCT (unmanipulated graft)                            | No                                                          | Direct selection of IFN-γ secreting T cells after incubation with recombinant pp65                        | Functional immunity detected in all<br>5 required antiviral agents but for shorter time than controls                                      |

MRD = matched related donor; CMV = cytomegalovirus; MMRD = mismatched related donor; MUD = matched unrelated donor; HSCT = hematopoietic stem cell transplantation; IFN-γ = interferon gamma.

### Tumor-Specific T Cells

Clinical immunotherapy targeting nonviral antigens is challenging especially when considering adoptive immunotherapy for malignant disease. Many of these tumor antigens are self antigens, and as a result of self-tolerance mechanisms, CTLs against these antigens are of low frequency, have low T-cell receptor avidity, or are anergic. In the allogeneic setting, preclinical studies have shown that it is possible to generate leukemia-specific CTLs using dendritic cells as antigen-presenting cells.<sup>104</sup> A report has detailed a patient with relapse CML who attained complete remission following infusion of leukemia-specific CTLs generated from the original allogeneic stem cell donor.<sup>105</sup>

In the autologous setting, dendritic cells pulsed with defined tumor peptide epitopes have been used as antigen-presenting cells. With this approach, CD8+ CTLs specific for the melanocyte differentiation antigens, Melan-A and gp100 peptides, could be generated from the PBMCs of HLA-A2-positive patients.<sup>106,107</sup> In 10 patients with metastatic melanoma who received autologous CTLs in conjunction with low-dose IL-2, three had mixed

responses and five had stable disease. The *HLA-A\*0201* melanoma line pulsed with Melan-A peptide analogue could also be used successfully as an antigen-presenting cell, obviating the need to generate dendritic cells from individual patients. In this study,<sup>108</sup> 10 patients with metastatic melanoma were infused with Melan-A specific CTLs followed by exogenous IL-2 and interferon-α. One achieved complete remission, three had partial or mixed responses, and three had stable disease. Limitations of this approach are that CTLs generated from peptide-pulsed antigen-presenting cells are predominantly, if not exclusively, CD8+ and that targeting a single peptide epitope increases the potential for tumor escape.

An alternative strategy, that circumvents some of the concerns regarding peptide pulsing, is to generate tumor-infiltrating lymphocytes (TILs), which can be screened for recognition of melanoma cells using an interferon-γ secretion assay, and then expanded with OKT3. Such TIL lines usually contain both CD4+ and CD8+ T cells and can be shown to have specificity for MART-1 or gp100 epitopes.<sup>109,110</sup> Clinical trials using TIL and high-dose IL-2 in nonlymphocyte-depleted patients resulted

in objective responses in roughly a third of patients with most responses being transient.<sup>111</sup>

## Genetically Modified T Cells

### T Cells Transduced with $\alpha\beta$ -T-Cell Receptor

An alternative strategy to target weakly immunogenic tumor antigen is to alter the antigen specificity of T cells by gene modification. T cells can be transduced with genes encoding a different antigen receptor: either physiologic  $\alpha\beta$ -T-cell receptor (TCR) heterodimers or artificial chimeric T-cell receptors.<sup>112,113</sup>

Several  $\alpha\beta$ -TCR heterodimers specific for tumor antigens have been cloned, either from autologous CTL culture<sup>114,115</sup> or from an *HLA-A\*0201* transgenic mouse model whereby high-avidity murine TCRs can be cloned.<sup>116,117</sup> These include the TCR recognizing MART-1 and gp100 melanoma/melanocyte differentiation antigens, the NY-ESO-1 CTA, and a p53 epitope expressed on approximately 50% of cancers of common epithelial origin.<sup>118</sup> Although some inefficiency from heterologous pairing between endogenous and transduced TCR chains is expected, primary T cells transduced with cloned  $\alpha$  and  $\beta$  TCR chains do mediate anti-tumor activity *in vitro*.<sup>114,116-118</sup> To investigate the anti-tumor ability of genetically engineered T cells *in vivo*, one group administered peripheral blood lymphocytes transduced with the genes encoding the alpha and beta chains of the anti-MART-1 TCR. After transduction, approximately 30% of the CD8+ T cells expressed the  $\alpha\beta$ -TCR heterodimer specific for MART-1. The genetically modified T cells were administered to 15 patients with metastatic melanoma and persistence of the T cells was seen in all patients for  $\geq 2$  months after infusion. Two of the 15 patients had anti-tumor responses and in both patients the T cells were still detected in the peripheral blood at 1 year after infusion. This study suggested that T cells expressing  $\alpha\beta$ -TCR heterodimers specific for tumor antigens may have therapeutic potential *in vivo*.<sup>118</sup> However, the main drawback of cloned TCR is HLA-restriction which limits its use to a subset of patients with particular HLA type.

### T Cells Transduced with Chimeric Antigen

Another strategy for the generation of tumor-specific T cells is the transduction of T cells with chimeric surface proteins that transmit TCR signals in response to target cells. Such proteins are composed of an extracellular domain (ectodomain) usually derived from immunoglobulin variable chains, which recognizes and binds target antigen. This domain is attached via a spacer to an intracytoplasmic signaling domain (endodomain) usually the cytoplasmic segment of TCR- $\zeta$  chain, which transmits an activation signal to the T cell. Genetic engineering of CTLs for redirected CD30-, kappa-, CD20-, and CD19-specific target cell recognition has been achieved and these approaches are in the process of being translated to the clinic.<sup>9,119-121</sup> However, chimeric receptor signaling produces only limited activation of the T cells, and clinical trials in patients with solid tumors have shown that T cells expressing transgenic antigen-specific chimeric

receptors have limited therapeutic activity, in part because engagement of the chimeric receptor alone is insufficient to sustain T-cell growth and activation.<sup>122</sup> One means of solving this problem is to transduce antigen-specific T cells rather than non-specifically activated cells and take advantage of the costimulation provided to the native TCR by antigen. This approach has been evaluated *in vitro* using CTLs specific for EBV, CMV, and influenza.<sup>123-125</sup> An alternate strategy is to provide additional costimulatory signals, and the signaling domain of the TCR has been combined with specific endodomains derived from different costimulatory molecules including CD28, 4-1BB, or OX40.<sup>126-128</sup>

### T Cells Transduced with Suicide Genes

As discussed above, the infusion of an unmanipulated T-cell product will contain alloreactive cells able to induce GVHD. One means of reducing this risk is to transduce the donor T cells with a suicide gene that can be activated if the recipient develops GVHD. The most commonly used suicide gene is herpes simplex virus thymidine kinase (HSV-TK) that renders transduced T cells sensitive to the cytotoxic effects of ganciclovir. HSV-TK-transduced T cells have been evaluated in several clinical trials<sup>129-131</sup> that have shown that GVHD can be effectively controlled by ganciclovir-induced elimination of the transduced T cells. However, the thymidine kinase suicide gene is virus derived and potentially immunogenic<sup>132</sup>; therefore, new nonimmunogenic suicide genes based on inducible Fas and caspase 9 have been developed.<sup>133-135</sup> In these constructs the Fas and caspase 9 molecules have been fused to a human FK506 binding protein to allow conditional dimerization. In both *in-vitro* and *in-vivo* studies, T cells stably transduced with a retroviral vector coding for the dimerizable gene can be selectively eliminated after the exposure to a chemical inducer of dimerization.<sup>133,135</sup>

## NK Cells

NK cells are effectors from the innate immune system that also mediate antiviral and antitumor immunity.<sup>136</sup> Unlike T cells, NK cells do not undergo clonotypic gene rearrangement in order to express antigen receptors. Through a complex array of receptors they are able to determine non-self from self. There are several classes of receptors, including killer immunoglobulin-like receptors. NK-cell receptors may be activating or inhibitory so that NK-cell activity is regulated by quantitative differences in cumulative inhibitory and activating signals. The presence or absence of the respective ligands on recipient cells determines if NK cells will be primed to kill the targets.<sup>137</sup> In the clinical setting of mismatched HSCT, donor vs recipient NK-cell alloreactivity has been associated with better outcome, particularly in patients with acute myeloid leukemia who undergo transplantation while in remission.<sup>138</sup> Several immunotherapy approaches using NK cells have been tested in the clinic and recent studies have shown that haploidentical NK cells infused after lymphocyte depleting chemotherapy can have antitumor effects.<sup>139</sup>

## Improving Cellular Immunotherapy Approaches

### Improving Ex-Vivo Generation

One of the limitations of adoptive immunotherapy is the time taken to generate sufficient numbers of antigen-presenting cells to generate antigen-specific CTLs. Several groups have evaluated if artificial antigen-presenting cells can serve as a substitute. A number of approaches have been used including grafting ligands for the TCR and costimulatory surface molecules onto mouse fibroblasts or beads.<sup>62,140,141</sup> A second limitation is suboptimal persistence of infused cells and several investigators have attempted to identify the optimal phenotype of infused T cells. It is likely that a product containing both CD4 and CD 8 cells and both effector and memory cells will be required.<sup>142-144</sup> It is therefore important to correlate in vivo function with the source of antigen-presenting cells and T-cell culture conditions.

### Lymphocyte Depletion to Improve Expansion of T Cells In Vivo

As discussed above, outside the setting of HSCT, poor lymphocyte survival or function has been a frequent limitation in immunotherapy studies. Several groups have evaluated whether administration of lymphocyte-depleting chemotherapy such as fludarabine or monoclonal antibodies may induce an environment that would promote the homeostatic expansion of infused T cells.<sup>109,145</sup> This strategy may also have a second benefit by depleting Treg cells. Clinical studies have already shown expansion of T cells specific for antigens expressed on solid tumors after lymphocyte depletion.<sup>146</sup>

### Genetic Modification of T Cells to Overcome Tumor Evasion Mechanisms

Tumor cells evade a transferred T-cell response by several mechanisms such as downregulation of MHC and costimulatory molecules and secretion of inhibitory cytokines by the malignant cell population.<sup>147</sup> One cytokine, which has devastating effects on CTL proliferation and function, is transforming growth factor-beta (TGF $\beta$ ), which is secreted by a wide variety of tumors as a powerful mechanism by which the tumor cells can escape the immune response.<sup>148,149</sup> The importance of this mechanism is illustrated by the observation that transgenic mice genetically engineered so their T cells are insensitive to TGF signaling are able to eradicate tumors.<sup>150</sup> Moreover, adoptive transfer of T cells transduced with a dominant-negative TGF $\beta$  Type II receptor (DNR) resulted in eradication of tumor cells.<sup>151</sup> Human in-vitro studies have shown that that DNR-transduced CTL were resistant to the antiproliferative effects of recombinant TGF $\beta$  and long-term expression of this construct had no deleterious effects on the function, phenotype, or growth characteristics of the transduced-CTL lines.<sup>152</sup> CTL expressing the DNR may, therefore, have a selective advantage in vivo in patients with TGF $\beta$ -secreting tumors. A study to test this hypothesis will open

shortly now that in-vivo safety studies in a murine model have been completed.<sup>153</sup>

## Regulatory Considerations for Adoptive Immunotherapy

In the United States, human cells, tissues, cellular and tissue-based products (HCT/Ps) that have been more than minimally manipulated (cultured ex vivo, transduced or activated ex vivo) are referred to as Type 351 products and must be prepared under good manufacturing practice (GMP) regulations to ensure their safety, purity, and potency. In cellular therapy, guidance from the Food and Drug Administration show GMP as a continuum, so that as an approach moves through Phase I and II clinical trials the application of GMP will become more rigorous, until in a Phase III study manufacture would be a fully validated process. A Phase III product would also need validated release criteria, which are test specifications designed to ensure that the product is sterile and pure, and has the desired functionality. In the United States new good tissue practice (GTP) regulations came into effect in May 2005 to cover HCT/Ps that were minimally manipulated (eg, were not cultured ex vivo, genetically modified, activated ex vivo, etc), and that were intended for autologous use, or use in a first- or second-degree blood relative. Cellular products that fall into this classification are referred to as Type 361 products and require manufacturing of the product under GTP regulations. In general, these cover personnel, procedures, facilities, environmental control and monitoring, equipment, supplies and reagents, recovery, processing and process controls, process changes, process validation, labeling controls, storage receipt, predistribution shipment and distribution, records, tracking, and complaints.

Implementation of the components of either GMP or GTP is a time-consuming and labor-intensive process that requires the development, implementation, and maintenance of numerous components. Professional societies, such as the Foundation for the Accreditation of Cellular Therapy and AABB have developed an accreditation process that takes into account GTP regulations and provides a framework around which compliance can be built. Both organizations have developed standards that are harmonized with regulatory requirements, so that accreditation by either organization assists with regulatory compliance.

The challenge of bringing adoptive cellular therapy protocols from the laboratory bench to the bedside has also been recognized by funding agencies, and the NIH has designated specialized centers for product assistance to provide consulting, manufacturing, and regulatory support to other research centers.<sup>154</sup>

## Conclusions

T-cell therapies have produced definitive benefits in the treatment of relapsed leukemia and lymphoma after transplantation

and EBV-associated malignancy. However, clinical studies have also identified limitations of such therapies including inadequate persistence or expansion. With increased knowledge of the optimal methods for generation of T-cell products, identification of additional antigen targets, and optimization of gene therapy approaches to enhance the function of adoptively transferred, adoptive immunotherapy strategies may find broader indications in the therapy of cancer and infections.

## Disclaimer

The authors have disclosed no conflicts of interest.

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Gene therapy, the use of DNA that encodes or corrects a gene for medical therapy, is achieved by transfer of genetic material to a patient's cells. Gene therapy can be achieved by repairing the defect of the endogenous gene (called targeting a gene) or by introducing additional genetic material into the genome. The specific manner by which gene therapy is performed varies significantly based on the disease to be treated, the gene to be inserted, the target cells of insertion, the vector used to transmit the gene, and the route of administration of the gene. This chapter discusses these variables as well as the current successes and challenges in the field of gene therapy.

Some of the many potential diseases that may be treated using gene therapy are listed in Table 60-1. It is hoped that the target diseases for gene therapy will eventually include most serious diseases for which the molecular etiology is well understood. Gene therapy may prove useful for gene replacement for congenital enzyme deficiencies or gene abnormalities. For treatment of cancer, gene therapy may be used to induce selective cancer cell death. Alternatively, cancer treatment could be achieved by using gene therapy to replace a missing tumor suppressor gene, to insert a chemotherapy drug-resistance gene selectively into normal cells, or to enhance the immunogenicity of cancer cells. It is also hoped that gene therapy will be developed to treat infectious diseases such as AIDS.

### Gene Therapy and Transfusion Medicine

Transfusion medicine is the science of administering cells or other biologic materials (eg, serum, isolated clotting factors) to treat patients. The growing field of gene therapy has affected transfusion medicine on several levels. First, gene therapy is introducing new biologicals for transfusion in the clinic setting. Gene therapy falls within the purview of transfusion medicine

**Table 60-1.** Diseases that May Be Treated Using Gene Therapy

| Disease Type             | Examples of Diseases                   |
|--------------------------|----------------------------------------|
| Gene deficiency/mutation | Hemophilia                             |
|                          | Cystic fibrosis                        |
|                          | Thalassemia                            |
|                          | Severe combined immunodeficiency       |
|                          | Gaucher's disease                      |
|                          | Sickle cell anemia                     |
| Cancer                   | Lack/mutation of tumor suppressor gene |
| Autoimmune diseases      | Rheumatoid arthritis                   |
| Infections               | AIDS                                   |

because it often requires the infusion of either gene-modified cells or direct administration of vectors that contain the therapeutic gene. As with blood components, the safety and efficiency of gene therapy agents must be optimized. Therefore, as gene therapy strategies are found to be effective in preclinical studies, quality control and infection control measures need to be developed for the clinical laboratory.

Second, hematopoietic cells are excellent targets for gene therapy trials. They are easily accessed and may be incubated ex vivo with the vectors containing the genetic repair material. Autologous marrow or peripheral blood stem cell (PBSC) transplantation already provides a relatively safe standard of care for some malignancies, and the circulation of these cells suggests that hematopoietic cells may be used as gene delivery systems even for nonhematologic diseases. Ideally, one needs only to infect the "stem" cells in order to get large-scale amplification and long-term expression of a transgene.

Third, gene therapy strategies are under development to treat several diseases that directly affect transfusion medicine, including hemoglobinopathies and clotting factor disorders. For example, hemophilia A, in which patients lack expression of functional clotting Factor VIII, is likely to be one of the first diseases to be treated successfully with gene therapy. This is because

functional Factor VIII protein product can be produced by any cells in the body, not necessarily the normal site of production in the liver, as long as the protein is secreted into the circulation. Also, the levels of circulating Factor VIII that are necessary for therapeutic efficacy need not be highly regulated as long as there is at least 5% of the normal physiologic level present.

## Vector Design

The many aspects that go into designing an ideal gene therapy vector are based largely on the specific disease to be treated, the target cells, and the therapeutic strategy. Three major considerations are which gene to insert, which vector to use, and how to administer the vector.

### What Gene Should Be Inserted and Where Should It Be Inserted into the Genome?

Based on the disease to be treated, the gene that will be inserted can confer a variety of attributes. The gene can provide a functional form of a missing or defective gene, it can be a gene designed to give the cell in which it is expressed a survival advantage, or it can act as a suicide gene designed to kill the cell in which it is expressed. See Table 60-2 for examples of each of these gene insertion strategies. For inherited diseases in which there is a nonfunctional gene product, such as cystic fibrosis, gene therapy approaches are being taken to add a functional gene. Similarly, for inherited diseases in which a gene is missing, such as thalassemia, the gene for the fully functional protein product is inserted. For cancer, the genes to be inserted can take one of several different forms. For example, one could insert a suicide gene selectively into the cancer cells. An example of one such “suicide” gene is HSV-TK (herpes simplex virus thymidine kinase), which can phosphorylate ganciclovir, which is then incorporated into the DNA, preventing its replication and thereby killing the cell. Alternatively, one could replace a missing or mutated p53 gene

with the wild-type gene, which could restore normal apoptotic (cell death) machinery to the cell.

In addition to determining which gene to administer, gene regulatory elements must be inserted in order to achieve appropriate gene expression levels. The process by which a gene is transcribed into messenger RNA (mRNA) and then translated into protein is controlled at multiple levels. For some therapeutic genes, the level of expression does not need to be highly regulated (eg, circulating level of clotting Factor VIII in hemophilia or adenosine deaminase levels). In contrast, other therapeutic genes, if expressed at either too high or too low a concentration, could be deleterious (eg,  $\beta$ -globin). If diabetes is going to be treatable by gene therapy, then insulin must be produced in response to elevated circulating glucose levels and reduced when the glucose level falls below normal levels. Every cell type in the body, although containing the same genomic DNA, presents a different pattern of gene expression. This process is controlled at multiple levels, the most common of which is transcriptional control by gene promoters, enhancers, and silencers in the specific context of a cell's nucleus. Promoters that are used only in specific cell types (eg, the insulin promoter in pancreatic cells) are referred to as cell-type-specific or tissue-specific promoters. Research has been focused on designing the regulatory domains of the transgenes (the promoters) so that the gene is either regulated as it would normally be in the cell with a tissue specific promoter, or regulated by an inducible promoter.

Another level of control for gene expression is to design gene therapy vectors that affect only specific target cells, so that the transgene is not expressed inappropriately in the body, and is not expressed by cells in which it would be deleterious. In the last few years, control of where a gene therapy vector may integrate in the genome has grown more critical. For example, in the case of recent clinical trials in X-linked immunodeficiency, discussed in further detail below, the therapeutic retroviruses integrated into a region of the genome, where they led to activation of proto-oncogenes that caused the patients to have leukemia. In addition the genome architecture adjacent to the proviral

**Table 60-2.** Genes Targeted in Gene Therapy to Date

| Gene Type                                | Disease                                                        | Gene                                                              | Target Cell                  |
|------------------------------------------|----------------------------------------------------------------|-------------------------------------------------------------------|------------------------------|
| Replacement of missing or defective gene | Thalassemia                                                    | $\beta$ -globin                                                   | Hematopoietic stem cell      |
|                                          | Sickle cell anemia                                             | $\beta$ -globin                                                   | Hematopoietic stem cell      |
|                                          | Severe combined immunodeficiency                               | Adenosine deaminase                                               | T lymphocyte                 |
|                                          | Cystic fibrosis                                                | Cystic fibrosis conductance regulator                             | Respiratory epithelial cells |
|                                          | Hemophilia                                                     | Clotting Factor VIII or IX                                        | Hepatocyte                   |
| Survival advantage                       | Chemotherapy for multiple forms of cancer                      | Multiple drug resistance                                          | Hematopoietic stem cell      |
| Suicide gene                             | Graft-vs-host disease caused by allogeneic donor T lymphocytes | Thymidine kinase gene<br>(kills cell when exposed to ganciclovir) | Hematopoietic stem cell      |
|                                          |                                                                | Cytosine deaminase<br>(kills cell when exposed to 5 FU)           | Hematopoietic stem cell      |

integration site can lead to silencing of transgene expression. The use of “insulating” genetic material at the extremity of the therapeutic cassette may prevent the ability of the inserted genetic material to perturb the activity of surrounding genes of the area of integration. Each of these concerns theoretically can be overcome if efficient gene targeting can be attained.

How Should the Gene Therapy Be Administered?

Gene therapy vectors can be applied to cells *ex vivo* and then returned to the patient. Alternatively, viral vectors that are replication incompetent (incapable of replication) and nonviral vectors can be directly administered to the patient for infection of target cells *in vivo*. So far, most animal and human clinical trials have used *ex-vivo* infection of patients’ cells with retroviruses. This allows for selection of virally infected cells before use in order to guarantee vector insertion.<sup>1</sup> The *ex-vivo* approach minimizes infection of unintended cellular targets, and because the cells are washed free of unincorporated viral vector before infusion, this approach significantly decreases the potential risks of exposing the patient to large amounts of viral vector. This approach has been used for hematologic disease in which the target cells can be harvested from patients and returned by intravenous transplantation. On the other hand, direct administration of vectors to the patient overcomes the difficulties of maintaining fully functional target cells *ex vivo*. However, because there are risks associated with transgene expression in unintended host cells, it is critical that the vector administered *in vivo* have target specificity or have little toxicity if expressed by cell types other than the intended target tissue. This *in-vivo* infection has been used mostly in clinical trials to treat patients with cystic fibrosis. Adenoviral vectors containing a normal version of the cystic fibrosis conductance regulator gene that is mutated in cystic fibrosis are inhaled and subsequently enter respiratory epithelial cells. Viral vectors have also been developed that can be administered either by intramuscular or by direct injection into the liver for the treatment of hemophilia.

What Vector Should Be Used?

The ideal vector should have no toxicity to the patient, should not cause an immune response, should be able to hold a large amount of genetic information (large gene-carrying capacity), should have target specificity or the ability to program target specificity, should infect target cells with a high efficiency, should have controllable gene expression (persistent versus inducible), and should have a controlled integration site that prevents nonspecific effects. The choice of which vector to use may also be based on the level and longevity of expression desired. Depending on the application, some genes need to be expressed over the long term in the individual (eg, replacement of a missing globin gene in thalassemia) and, therefore, must be incorporated into the genomic DNA of a cell population that will endure (the hematopoietic stem cell), and others may require only transient expression (eg, induction of tumor cell death) for which adenoviral vectors can be useful. Each application requires the design

Table 60-3. Common Viruses Used in Gene Therapy Research

| Family/Subfamily and Characteristics                                                                                                     | Specific Virus                                                                                   |
|------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|
| <b>Retroviruses</b> (single-stranded RNA, with maximal insert size of approx. 8 kb)                                                      |                                                                                                  |
| Oncoviruses                                                                                                                              | Murine leukemia virus<br>Spleen necrosis virus<br>Rous sarcoma virus<br>Avian leukocytosis virus |
| Lentiviruses                                                                                                                             | Human immunodeficiency virus, type 1<br>Human immunodeficiency virus, type 2                     |
| Spumaviruses                                                                                                                             | Foamy virus                                                                                      |
| <b>Adenoviruses</b> (double-stranded DNA, with maximal insert size of approx. 8 kb for first generation; up to 37 kb for new generation) | Adenovirus, type 5                                                                               |
| <b>Adeno-associated virus</b> (single-stranded DNA with maximal insert size of approx. 5 kb)                                             | Adeno-associated virus, type 2                                                                   |

of specific gene elements for insertion into specific vectors that will target appropriate levels of gene expression to the appropriate tissues.

Of course, no viral or nonviral vector yet achieves all of these goals; different vectors have different advantages and disadvantages. Many research laboratories are developing strategies to optimize current vectors to meet the criteria for an ideal vector. Provided below is an overview of the successes and remaining challenges in three of the most well established viral vector systems for clinical trials, retroviruses, adeno-associated viruses, and adenoviruses. Table 60-3 summarizes the salient features of these viral vectors. Nonviral techniques that are under development for gene therapy are discussed below as well.

Viral Vectors

Retroviruses

Retroviruses, the first gene therapy vectors to be used, generally have an enveloped single-stranded RNA genome and are approximately 100 nm in diameter. For applications using retroviruses, the therapeutic gene is inserted between the two long terminal repeats (LTRs) at each end of the retroviral genome and replaces three key normal viral genes (*gag*, *pol*, and *env*). Without these genes, the virus is no longer able to proliferate. The LTRs are critical for incorporating the therapeutic genes into the genomic DNA of the target cells and may also be required for expression of the inserted genes. The maximal insert size for the expressed gene(s) is approximately 8 kb, which can be quite limiting because the coding region for many genes is longer than 8 kb, and often more than one gene (eg, a therapeutic gene and

a selectable marker) may need to be included in the retroviral vector.

There are multiple types of retroviruses used in gene therapy research, including gamma retrovirus or oncoretroviruses, lentiviruses, and spumaviruses. To date, most retroviral vectors assessed for clinical trials have been based on murine leukemia viruses (MLVs). As described below, lentiviral vectors based on RNA sequences from human immunodeficiency virus (HIV) are better than oncoretroviruses at infecting nondividing cells. Although the therapeutic vector, in this case, is based on the HIV genome, these vectors do not contain the genes in the virus that can cause AIDS. The ability to transduce nondividing cells is associated with the viral genes (eg, gag matrix protein MA, the regulatory protein Vpr, and viral integrase) that efficiently shuttle the virus into the nuclei of target cells. Lentivirus can transduce primate<sup>2</sup> and human<sup>3</sup> hematopoietic stem cells (HSCs) more efficiently than the oncoretroviruses because the majority of HSCs are in G<sub>1</sub> or G<sub>0</sub> phase of the cell cycle. Recently, the human foamy virus (HFV) has been used as an alternative gene therapy vector to murine leukemia viruses and HIV-1 vectors. HFV is nonpathogenic, has the unique property of completing reverse transcription before entering its target cells, and infects multiple cell types including hematopoietic cells.<sup>4</sup> Although progress has been made in the last few years, the full cell biology of this virus is not yet well defined. Studies showing infection of nondividing cells<sup>5,6</sup> including unstimulated HSCs suggest that this vector system should be pursued more thoroughly. The unique capability of this virus to transduce metabolically inactive cells appears to be related to the fact that the foamy virus particles contain active reverse transcriptase and, as mentioned, the transcription of double-strand DNA is completed before the entry of the particles in the cell. Therefore, these viruses do not rely on the limited host nucleotide supply for their replication.<sup>7</sup>

### Optimization of Retroviral Vectors

Optimization of retroviral vectors should combine several features, which are described below.

#### Virus Inactivation

For safety reasons, all gene therapy vectors must be incapable of replication in the human host. By removal of the *gag*, *pol*, and *env* genes, replication-incompetent retroviruses (RIR) are produced. Because the genes removed are necessary for packaging the RNA genome into retroviral particles, special “packaging” cells are required to produce retrovirus for use in gene therapy. These packaging cells express the *gag*, *pol*, and *env* proteins because the three genes have been introduced into their genomes. When an RIR vector containing the LTRs and the packaging signal is introduced into such packaging cell lines, its RNA is packaged into infectious particles by the *gag*, *pol*, and *env* proteins. The DNA encoding viral *gag*, *pol*, and *env* is not contained within the viral particles produced, so that even though these retroviruses are capable of infecting target cells, they are not capable of replication. However, if some of the *gag*, *pol*, and

*env* sequences within the packaging cell genome share regions of DNA overlap with the retroviral coding region, there is a risk of recombination between the two, which could lead to the production of replication-competent retroviruses. MLV has the advantages of having the lowest overlap between viral elements and the genes inserted into packaging cells. In contrast, lentiviruses have a large portion of overlap in the N-terminus of the *gag* gene, and HFV requires the greatest overlap between coding sequence and RIR for generating potentially therapeutic viruses. Of note, the oncoretroviruses used for recent clinical studies were produced several years ago, and still contain large portions of the *gag* and *pol* genes, which increase the risk of making replication-competent viruses. Before any retroviruses can be used in clinical trials, however, each batch must be tested extensively in order to ensure that there are no replication-competent viruses present. Ongoing studies are focused on reducing the sequence overlaps without decreasing the titer of the viruses produced.

A step forward in the design of viral vectors was to create self-inactivating vectors by deleting the enhancer promoter sequences from one of the two viral LTRs containing the retroviral promoter and enhancer elements. Given the retroviral cycle, mutation of the transcriptional element at the 3' end of the virus does not affect the ability of the vector to infect cells and incorporate into the genomic DNA. However, after entry into the target cells, the 5' LTR, which is derived from the 3' LTR, also becomes mutated, and the LTR is incapable of promoting transcription. This approach greatly reduces the risk of creating replication-competent virus. The downside is that now the vector no longer has its own promoter, so it needs to be designed with another promoter that will drive transcription of the desired transgene once inside the target cells.

#### Efficiency

Some of the best target cells for in-vitro retrovirus infection are hematopoietic cells. Highly proliferative hematopoietic progenitors, measured by in-vitro colony-forming assays, have been infected successfully by retroviruses. The biggest obstacle to using retroviruses to infect hematopoietic stem cells is that oncoretrovirus do not infect nondividing cells, and hematopoietic stem cells cycle very slowly and are usually in G<sub>0</sub> phase of the cell cycle. The efficiency of infection of HSCs has been increased in recent years by addition of cytokines to the ex-vivo culture medium before and during retroviral infection in order to increase the number of HSCs in the cell cycle. However, such cytokines may also cause HSCs to differentiate so that they lose their long-term repopulating “stem cell” capacity. Retroviral infection and modification of mouse HSCs has been demonstrated using mouse marrow transplantation,<sup>8</sup> and efficiencies obtained have been very high with 80% to 100% of mouse stem and progenitor cells being infected with retrovirus. Infection of human HSCs is less efficient than murine cells.

In contrast to oncoretroviruses, lentiviruses and foamy viruses are able to efficiently infect quiescent cells and, therefore, appear to be more promising vectors for HSC-based gene therapy. Lentiviral vectors have a high efficiency of infection (up to 80%) in human

CD34+ cells from cord blood, cells in peripheral blood that have undergone granulocyte colony-stimulating factor (G-CSF) mobilization, and marrow cells.<sup>9</sup> Similarly, HFV is found to infect more than 80% of human CD34+ hematopoietic progenitors from cord blood.<sup>6</sup> Clinical trials using lentiviral vectors have been initiated for inherited diseases and HIV.

Experimental models for human HSCs, take advantage of the ability of human cells to engraft the marrow of immunocompromised animal hosts. These xenogeneic in-vivo models have been quite useful as preclinical systems. One such model uses sublethally irradiated nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. The human cells that maintain multilineage long-term engraftment in these mice are referred to as SCID-repopulating cells (SRC).<sup>10</sup> In a related immunodeficient mouse system, human CD34+ marrow cells were infected with an efficiency of approximately 10% by retroviral vectors, and these cells survived in the immunocompromised mouse hosts for at least 8 months.<sup>11</sup>

Specificity and Efficiency

The different degree of retroviral infection between mouse and human HSCs is caused by multiple factors that must be optimized in order to obtain high levels of infection. Much research has focused on two major differences between infection of mouse and human cells, 1) the percentage of stem cells in mitosis and 2) the surface expression of appropriate retroviral receptors. As alluded to above, in order for viral vectors to be expressed in the target cells, they must be able to enter the nucleus. For all viruses, except lentiviral vectors, this requires nuclear membrane breakdown, which occurs when a cell undergoes mitosis. Thus, quiescent, non-dividing cells are more difficult to infect with gene therapy vectors than are actively dividing cells. In mice, approximately 8% of HSCs enter the cell cycle per day in vivo, and about 75% are quiescent in G<sub>0</sub> phase at any time. It is estimated that human HSCs cycle less often than murine cells, and therefore a higher percentage of human HSCs are quiescent than are murine cells.<sup>12,13</sup>

The efficiency of retroviral infection is also highly dependent upon whether the retrovirus can bind to and enter the cell. Different retroviruses have different receptors. (A list of viral vectors and their receptors is given in Table 60-4.) For the retrovirus used most often in mouse cells, the receptor on the cell is CAT-1, an amino acid transporter.<sup>13</sup> In contrast, the retroviruses that have been used to infect human cells, called amphotropic retroviruses, bind to Pit-2, which is a phosphate transporter.<sup>14</sup> It is estimated that the CAT-1 retroviral receptor is expressed on a higher percentage of mouse HSCs than is Pit-2 on human HSCs.<sup>15,16</sup> Preselection of a subpopulation of hematopoietic cells that expresses high levels of the retroviral receptor and stimulation of receptor expression are two strategies that have been attempted to increase retroviral infection of human HSCs.<sup>17,18</sup> Higher levels of infection of human cells have also been achieved using “pseudotyped” retroviruses in which the envelope-encoding gene *env*, which encodes the ligand on the virus coat that binds to the target cells, has been replaced with a gene that is known to bind

Table 60-4. Viral Vectors and Their Receptors

| Virus                               | Receptor or Coreceptor                           |
|-------------------------------------|--------------------------------------------------|
| <b>Retrovirus</b>                   |                                                  |
| • Ecotropic murine leukemia virus   | CAT-1 (cationic amino acid transporter)          |
| • Amphotropic murine leukemia virus | Pit-2 (sodium-dependent phosphate transporter)   |
| • Human immunodeficiency virus      | CD4, CXCR4                                       |
| <b>Adenovirus</b>                   |                                                  |
|                                     | CAR (coxsackie virus and adenovirus receptor)    |
|                                     | $\alpha_v\beta_5$ or $\alpha_v\beta_3$ integrins |
| <b>Adeno-associated virus</b>       |                                                  |
| • Parvovirus B19                    | Red-cell-specific protein                        |
|                                     | Heparin sulfate proteoglycans                    |
| • AAV2                              | Fibroblast growth factor receptor 1              |
|                                     | $\alpha_v\beta_5$                                |

well to HSCs.<sup>19,20</sup> One protein that has been studied extensively is the gibbon ape leukemia virus (GaLV) envelope protein, which binds to a receptor called Pit-1.<sup>21</sup> Retroviruses pseudotyped with GaLV infect more than twice as many hematopoietic progenitors than the standard amphotropic retrovirus.<sup>22,23</sup> Gammaretrovirus and lentivirus are much easier to pseudotype than foamy virus.<sup>24</sup> After effective pseudotyping approaches are developed, this should allow for construction of vectors that are targeted to specific cells.

An additional level of specificity is to express the transgene on a tissue-specific promoter. For hematopoietic diseases, limiting expression of the therapeutic gene to differentiated cells rather than more primitive stem and progenitor cells could reduce the risk of genotoxicity of the vector. For example, vectors have been designed to treat erythroid disorders in which the  $\beta$ -globin gene is driven by a promoter that is active only in red cell precursors; preclinical studies using mouse models have shown promising therapeutic results.<sup>25</sup> A similar vector has also given promising results for a vector encoding human Factor IX.<sup>26</sup> Lineage-specific expression has also been achieved in T cells and B cells.<sup>27,28</sup>

Specific Chromosomal Targeting

All retrovirus vectors integrate randomly into genomic DNA. Based on the integration site of the viral DNA, expression of endogenous genes can be modified (genotoxicity). If this gene were a pro-oncogene, the activation induced by viral insertion could cause cancer. As discussed below, this is what occurred in a recent clinical trial in which four patients developed leukemia. The components that influence retroviral integration sites in the genome are not known. Ongoing studies are revealing general patterns of integration that characterize different vectors. For example, oncoretroviruses such as MLV integrate preferentially within a few kilobases upstream of transcriptional start sites in

DNA regions containing CpG islands (regions rich in cytosine residues next to and 5' to guanine residues). Lentiviruses also tend to insert near actively transcribed genes, but the integrations are not necessarily clustered near transcription start sites. Foamy viruses tend to integrate outside of genes, with both clusters and gaps.<sup>29,30</sup>

### Risks

The major risks associated with viral vector-based gene therapy are the generation of replication-competent viruses and integrations that lead to activation of oncogenes or silencing of tumor suppressor genes.

### Replication-Competent Viruses

Replication competent retroviruses develop either by recombination of the constituent parts of the vector system with endogenous viral sequence in the viral producer cell lines or by activation of endogenous proviral sequences. The significant risks of retroviral vectors for gene therapy were elucidated when primate studies were initially performed before 1992. CD34-selected marrow cells of the monkeys were infected with replication competent viruses from packaging cell lines. There were, however, some replication competent viruses that formed by rearrangement that had not been detected by the existing assays for replication-competent viruses. Upon infusion, three of eight monkeys subsequently developed lymphomas containing the active rearranged retrovirus.<sup>31</sup> As mentioned, investigators are optimizing the safety by creating self-inactivating (SIN) vectors, and minimizing the regions of homology between the vectors and the producer cells. Currently, all clinical trials require very stringent testing to guarantee that the retroviral vector to be used in humans is entirely replication incompetent.

### Genotoxicity (insertional mutagenesis)

Genotoxicity is caused by the insertion of the provirus in proximity to a tumor suppressor gene or pro-oncogene. The presence of a strong promoter of viral origin near a proto-oncogene can lead to transcriptional activation of the gene and ultimately the development of neoplasia. Unfortunately, this has occurred in a clinical trial for X-linked immunodeficiency.<sup>32-35</sup> More details regarding this trial are provided below in the Clinical Trials section. The best approach for minimizing oncogene activation may be the use of vectors such as lentiviruses and foamy viruses, which tend to integrate into areas of the genomes that are not oncogenic. However, additional evaluation of the possible risks associated with these vectors is required before they are ready for clinical trials.

No strategies are available currently for directing the integration of the transgenes in a safe, specific spot in the genome. Preliminary data suggest that an alternate approach, the addition of insulator sequences at each end of the transgene, can reduce the genotoxicity of retroviral insertion.<sup>36</sup> These *cis*-acting insulator sequences are designed to prevent the genome information contained inside the vector from interfering with the adjacent

endogenous neighboring genes. The best characterized chromatin insulator to date is derived from the chicken  $\beta$ -globin locus control region.<sup>37,38</sup> Developing vectors with tissue-specific promoters also addresses genotoxicity because these promoters allow improved spatial and temporal control of transgene expression.

### Challenges

The most important challenges for translating viral gene therapy into a compelling alternative treatment for hematologic diseases are 1) to create vectors with no adverse effects, 2) to ensure long-term therapeutic effects, and 3) to standardize protocols for highly controlled, efficient, high quality vector production.

### Prevention of Gene Silencing

Even if the insertion site causes no genotoxicity, expression of the therapeutic transgene can get silenced because of epigenetic modulation of the chromatin (eg, histone methylation) presumably because the host cell recognizes that the inserted DNA sequences are being expressed inappropriately. The elimination of a known silencing element within the U3 domain of the LTR, the use of a robust promoter (eg, PGK), the introduction of an intron sequence, and the incorporation of other transcriptional regulatory elements (eg, WPRE enhancers) are all strategies that have been evaluated to ensure long-term expression of the transgene. For example, SIN vectors, which lack functional domains of the LTR region, are less prone to silencing. These strategies are undergoing evaluation, and it is not yet clear that they will be useful for clinical studies.<sup>39</sup>

### Vector Production and HSC Modification

Additional problems with long-term retroviral infection of HSCs currently being addressed include establishing techniques for concentrating retrovirus onto the cells,<sup>40,41</sup> improving retroviral survival in vivo,<sup>42,43</sup> and optimizing infection efficiency into specific target cells.<sup>20</sup>

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## Adeno-Associated Virus

Adeno-associated virus (AAV) is a nonpathogenic, replication-defective, 4.68 kb, single-stranded DNA virus that requires external factors for infection. The name AAV was given because helper virus, usually adenovirus, is necessary for infection and replication. Advantages to AAV vectors over retroviruses include the ability to infect nondividing cells and insertion as DNA rather than RNA. This is a potential advantage over retroviral vectors because some genes, most notably  $\beta$ -globin, require the presence of introns for optimal expression, and these introns are likely to be spliced out when the retroviral RNA is reverse transcribed into DNA.

The inverted terminal repeats (ITRs) that are located at each end of the AAV genome are sufficient to package the single-stranded DNA into viral particles. When a gene therapy vector is made from AAV, the therapeutic gene can be inserted in



place of nearly the entire normal viral protein-coding domain (4.5kb) as long as the flanking ITRs remain intact. These vectors are packaged into viruses by supplying the required rep and cap AAV proteins separately. This is usually achieved by inserting both a vector plasmid encoding the therapeutic gene and a packaging plasmid encoding the *rep* and *cap* genes into cells also infected with adenovirus. Replication-incompetent AAV vectors are produced by lysing the cells to release viruses, and destroying the “helper” adenovirus with heat. In the absence of helper virus, AAV does not replicate and remains integrated in the genomic DNA of the producer cell. AAV is not pathogenic (approximately 80% of humans are seropositive for AAV type 2), making it suitable for gene therapy. AAV can infect nondividing cells and remain latent for prolonged periods in an episomal state. That is, AAV persists in cells without inserting into the genomic DNA (thus avoiding insertional mutagenesis) and does not trigger a robust immune response. Unlike the retroviruses, different naturally-occurring AAV variants, with more than 100 different capsid proteins, confer target cell specificity for AAV infection.<sup>44</sup> In addition, because of its genetic simplicity, AAV virus sequences can be mutated to increase the specificity of gene delivery; such modified AAVs are called recombinant AAVs. With repeated infections, AAV incorporates stably into the endogenous genome with a frequency of 1%, but most AAV remains in the episomal state. When AAV does integrate, it integrates randomly in the cells.<sup>45</sup> When more of the initial AAV genome is maintained, although the integration frequency is still quite low, the integration is targeted to a specific area of the genome (19q13.3), which is transcriptionally active. The targeted integration of the AAV is a unique characteristic among all known eukaryotic viruses, and, because the integration site appears to be nononcogenic, investigators are working on methods to maintain the targeted integration while making the AAV replication incompetent.

AAV vectors efficiently infect CD34+ human marrow progenitor cells. From 80% to 90% of CD34+ cells in suspension cultures and 50% to 95% of myeloid colonies differentiating in vitro from transduced CD34+ cells demonstrate AAV-induced transgene expression. A high percentage of transduction (up to 23%) has also been reported for CD34+ human cord blood cells.<sup>46</sup> In murine transplantation models with AAV-infected hematopoietic cells, no cytotoxic response to AAV occurs; mice transplanted with AAV-transduced marrow have long-term multilineage reconstitution,<sup>46</sup> and the transgene is still detectable after 6 months. In secondary mouse recipients (another test for whether self-renewing stem cells have been infected), a small but significant percentage (1%) of marrow cells were still transduced. A study in nonhuman primates demonstrated that 15 months after transplantation of AAV-modified autologous marrow cells, approximately 1 in 10 cells in the circulation was still modified. Modified cells were also detected in the marrow, and in specific subpopulations including granulocytes, B cells, and T cells.<sup>47</sup> Wang et al<sup>48</sup> developed capsid-modified, helper-dependent AAV vectors carrying the  $\beta$ -globin locus control region (LCR) to drive green fluorescent protein (GFP) expression. GFP expression was

detected in an erythroleukemia cell line. In primary CD34+ human cells, more than 40% expressed GFP at high levels.

AAV has been used in Phase I and II clinical trials for various diseases including Parkinson's,  $\alpha_1$ -antitrypsin deficiency, cystic fibrosis, and hemophilia B; however, no clinical trials using AAV-infected hematopoietic cells have been reported. Although this virus has been considered the safest vector in gene therapy (explaining the abundant ongoing clinical trials that are in place using this vector), the use of AAV in a clinical trial was recently associated with a severe adverse event.<sup>49</sup> In this clinical trial for the treatment of rheumatoid arthritis, a tumor necrosis factor, alpha (TNF- $\alpha$ ) inhibitor protein was delivered by an AAV vector that shuttled the gene into the joints. The trial, beginning in the fall of 2005, enrolled 127 patients without any serious adverse effects, but one patient during the second administration of the vector in the summer of 2007 developed a severe reaction to the vector that led to death in few days. This event is now, appropriately, under intense scrutiny. In addition, other investigators have shown that after several administrations of AAV in a mouse model of the lysosomal storage disease mucopolisaccharidosis VII, the vector integrated in a specific area of the genome on chromosome 12, which led to dramatic overexpression of the provirus and development of hepatocellular carcinoma.<sup>50</sup>

Another major problem with using AAV vectors is the highly variable efficiency of infection reported by different laboratories. One possible explanation for this variability is that different laboratories have different amounts of remaining intact adenovirus, which is coinfecting with the AAV. This is problematic because adenoviral vectors are replication competent. Improved techniques to effectively remove contaminating helper virus are currently being developed. Also, it has been difficult to obtain high titers of pure AAV. A “hybrid” between viral AAV vectors and liposomes (phospholipid-bound vesicles) has been proposed for gene therapy in which the AAV ITRs are used to flank the gene of interest in order to facilitate its insertion into the host genomic DNA, but rather than a viral particle, the DNA is packaged simply in a liposome carrier. Future studies in the AAV field are focused on 1) increasing the tropism of AAV for specific cells and 2) evaluating the “target” integration of the vector and the mechanism and proteins involved in this unique propriety of AAV, which could be therapeutically very important. In addition, deeper evaluation of the risks associated with the use of AAV is under way.

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## Adenoviral Vectors

Adenoviruses are double-stranded DNA viruses that are approximately 36kb in size. Four early genes are expressed at the beginning of the viral cycle, and are required for DNA replication and viral propagation. The late genes, which encode structural proteins, are transcribed after DNA replication. Unlike retroviruses, adenoviral vectors are quite stable in vitro, can be produced and concentrated to high titers, and can infect quiescent, postmitotic cells. After the adenovirus enters a cell, its

genome is released and maintained as an episome (circular DNA separate from the nuclear genomic DNA) in the cell. Because the adenoviral DNA does not incorporate stably into the genomic DNA of the cell, adenoviral gene expression is transient. The duration of expression of a gene on an adenoviral vector varies significantly based on the longevity and cell cycle status of the cell that is infected. Adenoviruses infect many different cell types, and there are more than 50 different types of adenoviruses that are associated with disease in humans. The diseases caused by adenoviruses are transient and usually are not serious unless the patient is immunocompromised. The cell membrane receptor for adenovirus fiber protein is called CAR for coxsackievirus and adenovirus receptor. The normal cellular function of CAR is not yet known. Similar to other types of viruses, interaction with a second membrane coreceptor is necessary for internalization of bound adenovirus. For adenoviruses, this second interaction is between the penton base of the virus and integrins on the cell surface including  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$ .

Adenoviral vectors have been tested extensively in models to treat cystic fibrosis, and have been used for modifying HSCs as well. The most widely used adenoviral vector is based on the Ad5 virus, belonging to adenovirus subgroup C. Human CD34+ hematopoietic stem/progenitor cells do not express CAR or the  $\alpha_v$  integrins at levels high enough for efficient Ad5 vector-mediated gene transfer. Therefore, when hematopoietic cells are exposed to adenovirus, there is only low-level transgene expression.<sup>51</sup> In contrast, human monocytes have relatively high levels of  $\alpha_v$  integrin and CAR.<sup>52</sup> Strategies to increase gene transfer efficiency of Ad5-based vectors include prolonged incubation of CD34+ cells with high concentrations of vectors, formation of virus-polycation complexes before infection, and cytokine stimulation of CD34+ cells. However, none of these approaches has led to significant improvement of transduction. More recent approaches have been focused on enhancing recognition of hematopoietic cells by adenoviruses by genetic or chemical modifications of the fiber/knob domain of the virus.<sup>53</sup> For example, investigators have replaced the entire fiber gene or the knob part of the Ad5 vector genome with the corresponding gene from Ad35, which does not require CAR expression on the target cell.<sup>54,55</sup> These modified adenovirus vectors show efficient (30-70%) gene transfer with little evident of cellular toxicity into hematopoietic cells, including CD34+ cells and primitive progenitor cells.<sup>56</sup> The infected populations were viable, and retained their ability to form colonies in short-term culture assays<sup>54</sup> as well as their ability to confer multilineage repopulation in vivo after transplantation into immunodeficient mice.<sup>53,56</sup>

The transient expression of this vector could have some potential applications for hematologic diseases such as transient overexpression of immune-response molecules for immunotherapies against leukemias or for transient expression of “stem cell genes” for expansion of HSCs in vitro without permanently affecting the genome of the cells and their progeny.

Ongoing challenges with adenoviral vectors include the immune response mounted by the host, the transient nature of

transgene expression due to lack of genomic insertion, and the inherent low affinity for hematopoietic cells. In clinical trials using adenoviral vectors to treat cystic fibrosis, problems arose because of an inflammatory response to the vectors. In addition, in cystic fibrosis there is a relatively low level of integrin expression on the airway epithelium.<sup>57</sup> Inflammation against adenovirus was also to blame in September 2000, when an 18-year-old patient with ornithine transcarbamylase deficiency, an inherited liver disease that causes life-threatening levels of ammonia to build up in the blood, became the first known death caused by gene therapy. This event demanded the attention of the public as well as that of regulatory agencies and committees that oversee and approve human gene therapy protocols. The patient, who had managed his disease with oral medication and a special diet, developed acute hepatitis after receiving an adenoviral gene therapy vector via the portal vein. Since that time, the Food and Drug Administration (FDA) has investigated several laboratories across the country and has found one failure to comply with National Institutes of Health (NIH) and FDA procedures. These findings raise the ethical quandary of whether to stop or slow the progress of gene therapy by tightening all regulations or to move ahead because patients with rare and untreatable diseases and their families want the trials to continue.

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## Nonviral Gene Therapy

Nonviral vectors for gene therapy are also undergoing intensive investigation.<sup>58</sup> Unlike viral vectors, nonviral vectors do not need to overcome the extensive immune mechanisms that destroy some viral vectors in vivo, and they do not have the risk of there being replication-competent forms of the vector. However, the application of nonviral gene therapy to humans has been severely limited by the poor efficiency of gene delivery to the cells. Several nonviral strategies under development are described briefly below.

### Episomal Plasmid DNA

The most common nonviral gene therapy approach is based on the transfer of plasmid DNA containing the correct cDNA sequence and regulatory elements of the gene of interest. The main challenges with this technology have been 1) the efficiency of gene delivery inside the cells, 2) appropriate shuttling of the vector from the cytoplasm to the nucleus where the plasmid is transcribed, and 3) the transient expression induced. Although plasmid DNA is not thought to be immunogenic, methylation of plasmid DNA may contribute silencing of transgene expression, which occurs when this strategy is tested in vivo. The DNA methylation issue can be overcome at least in part by making the therapeutic plasmids in cells that are incapable of methylating the DNA. However, the delivery of the plasmid DNA to the nuclei of the target cells is still a major concern. Approaches for increasing the passage of nonviral vectors into the cell, and into its nucleus include addition of 1) ligands for receptor-mediated endocytosis,

2) polypeptide sequences that assist DNA compaction, 3) endosomal disruption sequences, and 4) nuclear-import signals.

Among the technologies being developed for plasmid delivery are physical methods such as DNA microinjection, electroporation and gun injection, or chemical methods in which the plasmid DNA is combined with liposomal formulations or basic proteins and polymers such as polyethylenimine, to assist the plasmid cell entry by binding and/or enveloping the DNA through a charge interaction. Because of the transient expression of the transgene, the use of plasmid DNA for hematologic diseases has not been extensively studied.

### Integrated Exogenous DNA

Exogenous DNA, in circular as well as linear form, can spontaneously integrate into genomic DNA. Although this is a very rare event, there are some enzymes that have the capability to catalyze such reaction. This is the case for enzymes such as transposase, which are involved in the insertion of transposon elements into the genome, and site-specific integrases, which are found in bacteriophages and are able to first recognize unique nucleic acid sequences and then to mediate integration at that site. On the basis of this knowledge, investigators have developed nonviral vectors in which plasmid DNA is integrated into the genome of a target cell with the help of these enzymes. Two of these systems—the Sleeping Beauty vector (transposase-based method) and the phiC31 vector (integrase-based method)—are described below.

### Sleeping Beauty Vector

Transposable elements were not available for genome manipulations in vertebrates until recently, when an active element was resurrected from transposon fossils found in fish genomes.<sup>59</sup> This element, called Sleeping Beauty transposon, shows efficient transposition in cells of a wide range of vertebrates, including humans. The integration of a corrected copy of the gene of interest is achieved by transfecting the target cell with two plasmids: one containing the therapeutic gene flanked by the transposable element and the other providing expression of the transposase. The Sleeping Beauty transposon carrying the therapeutic gene is “cut” out of the plasmid vector by the transposase and then “pasted” directly into the chromosome. The efficiency of this process is quite high (up to 10%) and the tropism is for both dividing and nondividing cells. The vector does not appear to be toxic or to be associated with a dramatic immune response after repeated administration *in vivo*. The integration of the vector in the genome is random (transcribed and nontranscribed regions), with a general preference for adenine and thymine (AT)-rich DNA, microsatellite repeats, and a small bias toward genes and their upstream regulatory sequence.<sup>60</sup> The next challenge for this kind of technology will be to confer “specificity” for the integration site. Such targeted transposon integration at specific DNA sites is being tested by coupling a site-specific DNA-binding domain to the transposase.<sup>61</sup>

This system has been used for gene transfer into human CD34+ cord blood cells. When the vector containing GFP and the transposase was delivered to the cells via electroporation,

transgene integration reached a frequency of 6% and was stable for over 4 weeks. However, the gene transfer of more primitive cells (CD34+/CD38−) was less efficient because these cells were particularly sensitive to damage induced by the electroporation. Thus, although this vector may have an interesting potential application in gene therapy for hematologic diseases, the delivery of vector into primitive cells without damage is a significant challenge.<sup>62</sup> A Sleeping Beauty vector with erythroid-specific expression of  $\beta$ -globin is under investigation.<sup>63</sup>

### Recombinase

The phiC31 integrase is a site-specific recombinase originally isolated from a *Streptomyces* bacteriophage. The phiC31 integrase catalyzes precise, unidirectional recombination between its 30 to 40-bp attP and attB recognition sites in the genomic DNA. In mammalian cells, the enzyme also mediates integration of plasmids bearing attB into genomic DNA at sequences that have partial sequence identity with attP, termed pseudo attP sites. The integrase approach involves delivering the gene of interest as a plasmid also containing the integrase recognition site attB (40bp). The integrase pairs attB with the endogenous pseudo attP sites and catalyzes genomic integration. The system has been used successfully in gene therapy experiments in mouse liver and muscle, human skin, rat retina, and rabbit joints with an efficiency of 10% and with just a single copy integrated.

The great advantages of this vector in gene therapy is that pseudo attP sites are rare in mammalian cells.<sup>64</sup> A bioinformatics analysis revealed that phiC31 integrase-mediated integration into human pseudo attP sites was mostly (56%) distributed among 19 pseudo attP sequences. The integration sites were not close to cancer genes in the genome, suggesting that cancer risk may be minimal. However, a serious concern is that aberrant events were described, including large deletions and chromosomal rearrangements.<sup>65</sup>

To date, published data on using phiC31 for hematologic diseases have focused on optimization of phiC31 integrase-mediated integration into human T-cell lines.<sup>66</sup> The phiC31 integration sites in these cells were frequently located in intergenic regions on chromosomes 13 and 18 that may be preferred in hematopoietic cells. PhiC31 was used effectively to restore functional  $\gamma$ c protein in a mutant cell line, supporting the possible application of the phiC31 integrase-mediated genomic integration strategy as a gene therapy approach for treating SCID-X1.

### Gene Targeting

Gene targeting strategies attempt to directly correct the endogenous DNA sequence rather than introducing the therapeutic DNA by random insertion into the genome. In addition to allowing for potentially permanent correction of a gene disorder, such gene targeting avoids the risks associated with harmful mutations caused by the chance integration of the therapeutic DNA, prevents delivery of extra copies of the transgene, and allows for control of gene expression by the endogenous regulatory mechanisms. The technology is based on promoting homologous

recombination, which mediates the exchange between the endogenous (dysfunctional) target sequence and the corrected donor DNA sequence. Investigators have used several different targeting approaches including chimeric RNA-DNA oligonucleotides, short single-stranded oligonucleotides, triplex DNA, and small DNA fragments, in order to modify specific bases of various reporter genes, as well as different gene mutations associated with human disorders. These vectors, which vary in length and chemistry, in most cases are designed to be homologous to the target locus except for the nucleotides to be replaced.<sup>67,68</sup> Although each of these approaches has induced targeted homologous recombination, the frequency to date is too low for clinical applications.

### Zinc Finger Nucleases

Novel strategies are being developed in order to better target the site of homologous recombination. Based on the observation that double-strand breaks in the genome enhance gene targeting by homologous recombination, bifunctional zinc-finger (ZF) proteins are being designed to induce double-strand DNA breaks within specific DNA sequences. The two functional domains of the zinc-finger nucleases (ZFNs) are ZFs customized to recognize specific DNA sequences, and a nuclease domain. The amino acid composition of the ZF domains of proteins determines the specific DNA sequences to which they bind. ZFNs specifically designed to recognize different DNA sequences that flank a specific genomic locus, are fused in frame with the DNA cleavage domain from *FokI* nuclease. After two ZFNs dimerize, one that binds to a DNA sequence upstream and one that binds a different DNA sequence downstream of the target DNA, the nuclease will induce a double-strand break in that specific location. If donor DNA is present, homologous recombination will occur with a frequency up to 10%.<sup>69,70</sup> Although this technology is far away from translation to the clinic, ZFNs are able to stably and specifically modify primary human CD4+ T cells at the endogenous *IL2R $\gamma$*  locus with a frequency up to 6.6%.<sup>71</sup> Ongoing studies are focused on using this strategy for gene correction of sickle cell anemia, X-linked SCID, and Wiskott-Aldrich syndrome.

Recently, investigators have combined technology from lentiviruses and zinc finger proteins using the advantages of lentiviruses, their ability to enter the nucleus, with the site specific DNA nicks created by zinc finger proteins. They have inserted the cDNA for a zinc finger gene into a defective lentiviral vector that cannot incorporate into the DNA. Template (corrected) DNA plus the integrase gene are also introduced in order to achieve targeted gene correction.

## Clinical Protocols

### Basic Design of Clinical Trial Stages

In designing a clinical trial, different goals are defined for Phases I through IV. In Phase I trials, researchers test the treatment in a small group of people (usually 20 to 80) for the first time to

evaluate its safety, determine a safe dosage range, and identify side effects. Phase I studies are thus primarily concerned with safety and secondarily with the feasibility of the work proposed. In Phase II clinical trials, more people (100 to 300) are recruited in order to evaluate whether the therapy is effective and to further evaluate its safety. Most gene therapy protocols are currently in the Phase I or Phase II stages. In Phase III studies, larger groups of people (1000 to 3000) are treated in order to confirm that the therapy is effective, to monitor side effects, and to compare it with existing treatments. Factors involved in safety include production of sterile replication-incompetent vectors, prevention of adverse reactions, and minimization of patient immune responses against vector. Phase IV studies are postmarket trials that provide additional confirmation of the efficacy and safety of the regimen. Regulatory considerations for all clinical trials are assessed by multiple reviewers with different levels of oversight, including an institutional scientific review committee, institutional human investigation committee, institutional biosafety committee, the NIH recombinant advisory committee, and the FDA.

### Clinical Gene Therapy Successes and Failures

Several clinical trials of gene therapy for hematologic diseases have been completed. In all of these trials oncoretroviruses were used and a functional LTR promoter drove expression of the transgene. Overall, approximately 20 patients have been treated, and 80% are healthy and have recovered from their genetic defect.

### Clinical Trials for X-Linked Severe Combined Immunodeficiency

The first success in gene therapy was achieved by a French group who treated 10 children with X-linked SCID, in which there is a complete lack of functional T and natural killer (NK) lymphocytes in affected boys because of mutation of the gene encoding the shared subunit of the receptors for interleukins 2, 4, 7, 9, and 15.<sup>33,72,73</sup> A murine retrovirus vector was used to introduce the cDNA for the missing receptor *ex vivo* into autologous CD34+ marrow cells, and the cells were returned to the patients. Because of the survival advantages of the corrected HSCs over the endogenous defective cells, preconditioning was not necessary to obtain long-term engraftment in these patients. Circulating T and NK cells that contained the therapeutic transgene were detected in nine of 10 patients and the functional activity of the T and NK cells were the same as in normal unaffected children for at least the first 2.5 years following gene transfer. However, four of the 10 patients developed leukemia caused by insertional mutagenesis of the proviral vector near the proto-oncogene LMO2.<sup>32</sup> Unfortunately, one child died of leukemia; the other three patients are responding well to antileukemic therapy.<sup>74</sup>

At the same time, a very similar clinical trial was performed by a British group in which four children<sup>75</sup> and two adults<sup>76</sup> affected by X-linked SCID, were treated with autologous CD34+ marrow HSCs that were transduced with a gammaretrovirus (almost

identical to the vector used in the other clinical trials) encoding the corrected sequence for the receptor. In this study, the four children had restoration of functional immunity whereas the treatment was not effective in the older patients. To date, at least one patient treated in the British trial has developed leukemia. No reason is yet known for the different incidence of leukemia in the French and British trials; more time has elapsed since the French trial was performed so the comparison is not yet final.

### Clinical Trials for Adenosine Deaminase Severe Combined Immunodeficiency

Retroviral vectors containing the missing adenosine deaminase (ADA) gene were used to transfect autologous cord blood cells, which were then returned to three newborns who had been diagnosed with ADA deficiency in utero. Using in-vitro retroviral infection protocols that achieved levels of infection of 5% to 40% of hematopoietic stem and progenitor cells, the patients had low percentages (0.01 to 0.001%) of detectable circulating blood cells containing vector sequences after 1 year.<sup>77</sup> In these human studies, no replication-competent retrovirus has been detected. After 5 years, the authors reported that there were still some circulating cells that contain the transgene. Because these children are being treated with an oral form of pegylated-ADA (the standard of care for the disease), the potential therapeutic effect of the inserted transgene cannot be assessed.<sup>78</sup> Aiuti et al<sup>79</sup> have performed more recent clinical trials for ADA deficiency using an improved protocol for gene transfer into HSCs. In this clinical trial, the patients were not treated with pegylated-ADA and, to provide an initial engraftment advantage for the modified HSCs, patients received a low intensity, nonmyeloablative conditioning regimen using busulfan. Unlike X-SCID, the corrected cells in ADA deficiency do not have a strong survival advantage. Autologous CD34<sup>+</sup> marrow cells were transduced with the retroviral vector ex vivo, and infused into the patients.<sup>79</sup> After 47 months, all of the patients were healthy and cured from the genetic immunodeficiency associated with ADA deficiency.<sup>80</sup> Similar success was also recently obtained in another ADA-SCID patient treated with autologous CD34<sup>+</sup> cells transduced with ADA-expressing gammaretrovirus. As in the trials conducted by Aiuti et al, the patient received a nonmyeloablative conditioning regimen. However, in this case, the patient was undergoing pegylated-ADA treatment, which was stopped just before transplantation.<sup>81</sup>

In all of these clinical trials the vector used and the technical procedures for preparing the HSCs were very similar. A recent analysis of the different viral insertion sites in the genomes of patients from the three clinical trials<sup>82</sup> showed that the proviral insertion was similar in all three trials, and was not random but clustered near promoters of genes in GpC islands. No significant differences were found in the viral insertion sites between the patients who did or did not develop leukemia in the French trial.<sup>74</sup>

### Clinical Trial for Granulomatous Disease

To date, two patients affected by chronic granulomatous disease (CGD), characterized by neutrophil dysfunction with impaired oxidative antimicrobial activity, have been treated with gene therapy. As in the ADA-SCID trials, autologous HSCs were transduced with a gammaretrovirus encoding a functional copy of the transgene called gp91(phox), and the patients were treated with nonmyeloablative conditioning before infusion of the genetically modified cells. The patients had a robust amelioration of symptoms including clearance of infection associated with engraftment of corrected cells.<sup>83-85</sup> A careful evaluation of insertion sites and clonality analysis, revealed that although the initial marrow reconstitution was polyclonal, with time there was selection for cells having the provirus inserted in the promoter of genes involved in survival and cells division. Both patients showed insertions in the MDS-Evil locus, which is known to be associated with leukemia. One patient lost the transgene expressing cells and died of infection and, at last report, the other has remained well, without progression to leukemia.<sup>86</sup>

### First Clinical Trials Using Lentivirus Vectors

Lentiviruses are extremely promising vectors for gene therapy. The first Phase I clinical trial using this vector has recently been performed for the treatment of five patients with chronic human immunodeficiency virus (HIV). In this case, the vector is not used for delivering a corrected copy of a gene but, rather, for transcribing an antisense RNA sequence against the HIV envelope with the goal of impairing viral infection. Autologous CD4<sup>+</sup> cells were expanded ex vivo, transduced with the lentivirus vector, and infused into the patients. The treated patients showed an increased cellular response to HIV and improved the memory response of T cells. In one of the patients, the lentivirus-based gene therapy led to a robust antiviral response. At 21 to 36 months after treatment, there was no evidence of genotoxicity from insertional mutagenesis.<sup>87</sup>

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## Designing a Clinical Vector Laboratory

Gene therapy holds much promise for the treatment of malignant and nonmalignant diseases. But, just as there is the potential for benefit, there is also the potential to cause harm. Safety measures are being put into place at all levels from the basic science laboratories and centers where the work is being tested to the establishment of federal regulations designed to safeguard the patients, the people working with the transgenes, and the general public. The clinical stem cell laboratory, where gene therapy vectors are applied to the cells ex vivo, must be compliant with very stringent regulations designed to maintain safety and sterility. These complex and thorough regulations need to be followed in order to be within the limits of current good manufacturing practice (cGMP) regulations. By definition, cGMP regulations are “methods to be used in, and the facilities or controls to be used for, the manufacture, processing, packing, or holding

of a drug to assure that such drug meets [safety] requirements ... and meets the quality and purity characteristics that it purports or is represented to possess.”<sup>88</sup> The cGMP requirements are put into place to ensure that all procedures are performed in as safe and controlled a manner as possible. All of the general principles of cGMP are outlined by the FDA in the Code of Federal Regulations (CFR) in 21 CFR 210 and 211, 21 CFR 600s, and 21 CFR 820. Some of the key elements of cGMP encompass raw materials, maintenance of equipment, complete standard operating protocols, validation, record-keeping, and personnel training. Several professional organizations including the Foundation for the Accreditation of Cell Therapy and AABB have voluntarily established comprehensive standard-setting and accreditation programs that encompass all phases of cell collection, processing, and transplantation. Additional standards specifically for gene therapy are under constant development and improvement by the United States Pharmacopoeia (USP), the USP Biotechnology and Gene Therapy Subcommittee, and its Advisory Panel on Gene and Cell Therapies.<sup>88</sup>

## Summary

Gene therapy continues to offer much promise for the treatment of genetic and acquired diseases. Gene therapy has been used to treat patients with X-SCID, ADA-SCID, CGD, and HIV, and applications to other diseases are currently being tested in clinical trials. Transfusion medicine laboratories are currently involved in clinical gene therapy trials and, in the future, when these therapeutic modalities are approved, will oversee what may be “routine” administration of gene therapy vectors and vector-infected cells. This oversight requires extensive knowledge of cGMP as well as risk prevention/infection control in gene therapy.

## Disclaimer

The authors have disclosed no conflicts of interest.

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The fields of tissue engineering and regenerative medicine have evolved into a unified discipline with an interdisciplinary approach being a large part of the merger. The first use of the term tissue engineering in the public domain was at the proceedings of the Granlibakken workshop in 1985<sup>1</sup>: "Tissue engineering is the application of principles and methods of engineering and life sciences toward fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve tissue function." This was a broad definition that focused on the incorporation of living cells into acellular, related structures as a source for tissue replacements. In addition to providing a definition of tissue engineering, the basic components necessary to create a tissue-engineered product were identified. These components included a cell source, a method to induce specific tissue growth, and a biomaterial to act as a scaffold.<sup>2</sup> The principles of tissue engineering have been translated to clinical applications with the Food and Drug Administration (FDA) approval of products in the areas of skin substitutes, bone formation, and cartilage. Tissue engineering has the potential to revolutionize medical care by replacing diseased or damaged tissue without the use of conventional organ or tissue transplantation.

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**Overview of Tissue Engineering**

A cell source or method that can be used for all cell lineages and has the required characteristics to make it safe, reproducible, and without any immunologic rejection has not been developed. The options for a cell source are primary autologous, allogeneic, xenogeneic, or stem cells. Cells can be cultured with low rates of bacterial contamination through the use of antibiotics

and sterile technique. However, concerns about xenogeneic contamination remain if feeder cells or sera from other species are used in the cell culture conditions. An alternative to primary tissue is to induce progenitor cells to differentiate into a specific cell type. Difficulties associated with the usage of primary tissue often include the need for an operative procedure, inability to obtain sufficient normal cells, inability of cells to proliferate, and the presence of cells with the same genetic predisposition for development of a disease. Another complication of primary cell sources is the need to efficiently isolate a specific cell lineage with low contamination rates from other cell types. Cell sorting methods, such as magnetic or fluorescent cell sorting, provide a reliable method if the cell type has specific membrane proteins with known antibodies.

**Stem Cell Sources**

Stem cells are an attractive cell source for tissue engineering applications. Adult stem cells or human embryonic stem cells each offer their own set of advantages and challenges. Adult stem cells can typically be harvested by less invasive procedures from marrow, fat, or skin. Because these cells are autologous, no immunosuppressive medications are needed when the tissue is implanted in the donor. Purification difficulties and a reduced differentiation potential of adult stem cells as compared to embryonic cells, however, prevent use of these cells for all applications. Adult stem cells do not involve human embryos or any of the ethical controversies related to the collection of embryonic cells. The potential use of embryonic stem cells has created a national debate over ethical issues, because an embryo must be destroyed to harvest the cells. In the United States a limitation currently exists on public funding but not on private funding for human embryonic stem cell research.

**Scaffolds**

A scaffold should enable the creation of a three-dimensional formation of tissue in vitro and in vivo. Properties for the ideal tissue-engineered scaffold are the support of cell attachment, ability for the cells to proliferate and differentiate, allowance for

diffusion of nutrients and waste, and ability to simulate mechanical properties found *in vivo*.<sup>3</sup> Biomaterials can be categorized as synthetic polymers, natural materials, or inorganic matter; they can also be classified as being biodegradable or permanent.<sup>4</sup>

The preference for tissue-engineered scaffolds has been on biodegradable natural materials and polymers. Natural materials include collagen, gelatin, fibrinogen, dextran, glycosaminoglycans, and chitin. Examples of biodegradable synthetic polymers are polylactic acid (PLA), polyglycolic acid (PGA), and polycaprolactone (PCL). The properties of natural materials are their similarity to the native biologic environment, minimal toxicity, and degradation by natural enzymes.<sup>4</sup> The limitations of natural materials include batch-to-batch variability from different animal sources, and potential immunologic reactions if materials are of xenogeneic origin. The most commonly used FDA-approved biodegradable synthetic polymers are polyglycolic acid and polylactic acid. These synthetic polymers are degraded by hydrolysis. Copolymers of PGA and PLA can be designed in specific ratios to match the degradation process for the individual tissue requirements.<sup>4</sup>

Scaffold fabrication techniques provide a variety of methods to form a structure, alter the mechanical properties, and change the degradation rates. Fibers can be woven or knitted to form structures with controlled pore sizes.<sup>5</sup> The molecules self-assemble under the appropriate conditions. Natural materials, such as components in the extracellular matrix, often are capable of forming scaffolds by self-assembly.<sup>5</sup> Solvent casting and particulate leaching techniques involve dissolving a polymer in a solvent, and then casting into a mold filled with a porogen (eg, sodium chloride or gelatin). Gas foaming is a process wherein a polymer is prepared by compression molding, or where the gas, for instance CO<sub>2</sub>, is dissolved into the polymer melt. The structure is exposed to high pressure CO<sub>2</sub>; then, as the pressure is reduced, pores remain when the CO<sub>2</sub> evacuates.<sup>3</sup> Electrospinning is a method in which a polymer forms nanofibers as an electrified jet is deposited on a metallic collector.<sup>3</sup>

## Skin Tissue Engineering

The field of skin tissue engineering has developed in response to the clinical need for skin substitutes. The clinical applications that skin substitutes have focused on are burn injuries and chronic wounds. The estimated incidence of deaths from fire and burns in the United States is 4500 each year, and there are approximately 45,000 hospitalizations per year to treat burn injuries.<sup>6</sup> Chronic wounds that require skin substitutes are caused by arterial occlusive disease, venous disease, and pressure ulcers. These wounds affect more than 2 million people in the United States.<sup>7</sup> Although the need for skin substitutes in this group is less acute than in the burn group, the morbidity as a result of these chronic wounds is significant. For patients with nonhealing lower leg ulcers, such wounds can eventually lead to an amputation.

## Structure and Function

The structure of skin consists of the surface epidermis and the deeper layer of connective tissue, the dermis. The epidermis is mostly keratinocytes, which are responsible for the barrier function and strength of the epidermis. The connective tissue layer of the dermis contains fibroblasts, few adipose cells, blood vessels, mast cells, nerve endings, hair follicles, and glands. The main component of the connective tissue matrix is collagen, with other matrix molecules such as elastin and reticulin. The skin functions to provide a barrier between the body and the external environment, and to provide sensation, temperature regulation, and vitamin D production. The most important function of the skin as related to skin substitutes is for the epidermis to restore the skin barrier.

The origins of the field of tissue engineering derived from the establishment of skin substitutes. The early leaders in this field in the 1980s were Burke and Yannas at the Massachusetts General Hospital and Massachusetts Institute of Technology.<sup>8</sup> They developed a bilayer artificial skin substitute with a temporary silastic epidermis and collagen dermis that could support the growth of dermal fibroblasts from the wound bed.<sup>8</sup> Other investigators developed similar methods using allogeneic keratinocytes with collagen gel or on PGA mesh.<sup>9</sup> The first skin substitutes were approved by the FDA in the late 1990s for applications of venous leg ulcers and for temporary covering of partial-thickness burns.

## Design Goals for Skin Substitutes

An intact epidermal layer provides a barrier function to prevent fluid losses and to inhibit infection. An ideal skin substitute will permanently heal into the wound bed to replace the dermal and epidermal layers in a timely manner, to form a desirable cosmetic result, to have minimal risk of infection or immunologic rejection, and to have a low cost. The standard material for covering large burn wounds is an autologous split-thickness skin graft. Autologous skin grafts function to permanently heal into the wound bed, contain an epidermal and dermal component, and have no risk of immunorejection. Skin substitutes are a viable alternative to autografts because they are readily available, do not require donor site harvest, and allow for better healing than dressing changes alone. The skin substitutes provide a temporary coverage over the wound; eventually, they get rejected or require a secondary thin epidermal autograft. Because most of the skin substitutes do not heal into the wound, they promote healing by stimulating cytokines and growth factors in the wound bed to recruit and direct host cells to repair the wound site.<sup>10</sup>

Early alternatives to the standard of autologous skin grafts were allogeneic skin grafts and porcine xenogeneic skin grafts. Allogeneic skin grafts have been the preferred material for temporary coverage as a skin substitute over xenografts. The beneficial properties of the allogeneic skin graft are that it prevents desiccation of the wound, prevents fluid losses, has a dermal component, and stimulates repair of the wound bed for eventual

placement of an autograft.<sup>11</sup> Porcine skin grafts are temporary skin grafts that have similar properties to allogeneic skin grafts, with the addition of being more readily available and cheaper. Because allogeneic and xenogeneic grafts do not incorporate into the wound, these grafts carry the potential risk of infection and eventual immunorejection.<sup>11</sup>

### **Acellular Skin Substitutes**

The group of acellular skin substitutes includes Biobrane, AlloDerm, Integra, and TransCyte. Biobrane (UDL Laboratories, Rockford, IL) is a synthetic bilaminar skin substitute that was introduced in 1979. The outer layer is silicon rubber, while a secondary layer consists of a nylon mesh that is covalently bound to Type I porcine collagen.<sup>4</sup> The outer layer silicon pore size allows for drainage of exudate and permeability to topical antibiotics, but the small pore size acts as a barrier to bacterial invasion.<sup>4</sup> Additional properties of Biobrane are adherence to clean wounds, stimulation of ingrowth of cells from the wound bed, ease of application, and a shelf life of at least 3 years. Biobrane may be used for superficial partial-thickness burns, over a meshed autograft, and in donor sites. Biobrane has demonstrated a reduced time to re-epithelialization when compared to traditional therapy of topical antibiotics and dressing changes for burn injuries.

AlloDerm (LifeCell, Branchburg, NJ) is a dermal skin substitute that removes epithelial cells from harvested human skin to leave an acellular dermal matrix. AlloDerm is approved for treatment of partial to full-thickness burns and chronic wounds. AlloDerm has similar properties to allogeneic grafts, except the immunologic response is reduced because of the processing of the tissue.<sup>12</sup> The clinical use of AlloDerm for partial-thickness or full-thickness burns is similar to that of the dermal component, but an ultra-thin autograft is needed over the AlloDerm. The advantage of the ultra-thin autograft is that it reduces the morbidity or loss of donor site grafts, due in particular to scar formation. It has been shown that an acellular allograft with a thin autograft produces equivalent healing rates at 14 days in comparison to split-thickness skin grafts.<sup>13</sup> Further case studies have demonstrated high success rates of AlloDerm with a split-thickness autograft.<sup>14</sup>

Integra (Integra Life Sciences, Plainsboro, NJ) is a bilayer skin substitute that was approved in 1996 for use in partial-thickness and full-thickness burns. The dermal component is a biodegradable collagen-chondroitin-6-sulfate matrix with an epidermal layer that is a thin silicone elastomer. The thin silicone layer acts as a barrier to bacteria and controls moisture loss. The dermal component encourages the adherence and growth of the wound bed. After 2 to 3 weeks when a neodermis has formed, the thin silicone layer is removed and then covered with a thin autograft.

TransCyte (Advanced Tissue Sciences, La Jolla, CA) is also a bilayer skin substitute that was approved in 1997 for temporary coverage for partial-thickness or full-thickness burns with autografting. The outer epidermal component is a silicone thin

film with an inner dermal layer consisting of a nylon mesh coated with porcine dermal collagen with ingrowth of neonatal human fibroblasts. The material is cryopreserved, which destroys the metabolic activity of the fibroblast cells, but the tissue growth factors and matrix remain intact. The growth factors and complex matrix components may create an environment more favorable for the adherence and migration of fibroblasts. TransCyte has demonstrated faster healing times and reduced scar formation in comparison to topical antibiotics in partial-thickness burns.<sup>15</sup>

### **Allogeneic Skin Substitutes**

The allogeneic skin substitutes that are FDA-approved include Apligraf, Dermagraft, and OrCel. Apligraf (Organogenesis, Canton, MA) is a bilayer structure with Type I collagen gel and allogeneic fibroblasts that form a dermal equivalent, onto which allogeneic keratinocytes are seeded to provide an epidermal layer. Dermagraft (Dermagraft, Westport, CT) is a single-layer skin substitute using allogeneic fibroblasts cultured onto a bioresorbable glycolic acid scaffold. OrCel (Forticell Bioscience, New York, NY) is a bilayer material with fibroblasts cultured in a porous sponge as the dermal layer with keratinocytes seeded on the nonporous side of the matrix. Although the fresh form of OrCel is available, a cryopreserved formulation is in clinical trials. The mechanism of wound healing for these skin substitutes is not clearly understood, but they seem to stimulate epithelialization by the fibroblasts' release of growth factors and extracellular matrix to provide recruitment of endogenous cells.<sup>16</sup> The function of the allogeneic fibroblasts likely contributes a temporary response to the wound healing, because at 4 weeks after treatment, only two of 10 patients had Apligraf DNA in the wound site.<sup>17</sup> Apligraf, Dermagraft, and OrCel have all shown improved rate of wound closure when used for chronic ulcers in comparison to conservative treatment with dressing changes.<sup>18,19</sup> The primary application for the allogeneic skin substitutes is for the treatment of chronic ulcers or acute wounds, with limited use for partial-thickness or full-thickness burn injuries. Currently available skin substitutes are listed in Table 61-1.

The current skin substitutes constituted the first step in using tissue-engineered products for clinical applications. These skin substitutes have demonstrated some of the potential successes and challenges that remain to create an ideal tissue-engineered equivalent. The limitations of the existing materials are related to the allogeneic cells that seem to function by recruiting host cells for wound healing instead of primarily contributing to wound healing. Cultured epidermal autograft techniques are not suitable for widespread application because only the epidermis can be created, without a true bilayer structure. The results of the cultured epidermal autografts have been disappointing because they are fragile, require a dermal layer, and require weeks to grow in culture.

The future of skin substitutes may incorporate adult mesenchymal stem cells (MSCs) and gene therapy strategies for optimization. The design of a tissue-engineered skin graft could contain

**Table 61-1.** Clinical Skin Substitutes

| Reference                                                         | Skin Substitute                          | Application                                                  | Result (time to healing)                                            |
|-------------------------------------------------------------------|------------------------------------------|--------------------------------------------------------------|---------------------------------------------------------------------|
| Kumar <sup>22</sup> /Barrett <sup>23</sup>                        | Silver sulfadiazine                      | Pediatric partial-thickness burn                             | 11.2-16.1 days                                                      |
| Kumar <sup>22</sup> /Barrett <sup>23</sup><br>Still <sup>24</sup> | Biobrane                                 | Pediatric partial-thickness burn donor site                  | 9.5-9.7 days<br>18.4 days                                           |
| Calicut <sup>14</sup>                                             | AlloDerm over split-thickness skin graft | Deep partial or full-thickness burn                          | 26/27 graft take                                                    |
| Veves <sup>19</sup>                                               | Apligraf                                 | Chronic ulcers                                               | Heal % at 12 weeks:<br>Apligraf, 56%<br>Dressing, 38%               |
| Blight <sup>25</sup> /Still <sup>26</sup> /Clugson <sup>27</sup>  | Culture epithelial autograft             | Full-thickness burn                                          | Heal %: 15-80%                                                      |
| Purdue <sup>28</sup> /Martson <sup>18</sup>                       | Dermagraft                               | Full-thickness burn—temporary coverage; diabetic foot ulcers | 95% take<br>Heal % at 12 weeks:<br>Dermagraft, 30%<br>Dressing, 18% |
| Heimbach <sup>29</sup>                                            | Integra                                  | Full-thickness or deep partial-thickness burn                | Take Integra: 76%<br>Take epidermal graft: 88%                      |
| Still <sup>24</sup>                                               | Orcel                                    | Donor site; venous ulcer                                     | 13.2 days<br>Heal % at 12 weeks:<br>Orcel, 59%<br>Dressing, 36%     |
| Kumar <sup>22</sup> /Noordenbos <sup>15</sup>                     | TransCyte                                | Pediatric partial-thickness burn; partial-thickness burn     | 7.5 days<br>11 vs 18 days                                           |

autogenous cells in a bilayer structure with a dermal and epidermal component. The use of marrow MSCs cultured with autogenous dermal fibroblasts may enhance epidermal regeneration.<sup>20</sup> Gene therapy can be used to upregulate the delivery of growth factors or cytokines to expedite the regenerative process. In animal models, gene delivery with epidermal growth factor has been shown to dramatically accelerate healing.<sup>21</sup> However, for wound healing the upregulation of growth factors should be transient, because continued gene expression might result in adverse effects. The advancement of skin substitutes and a better understanding of the biology of wound healing will likely lead to the development of rationally based designs to specifically treat the pathology of the wound.

## Blood Vessel Tissue Engineering

Cardiovascular disease is the leading cause of mortality in the United States.<sup>30</sup> More than a million arterial bypass procedures performed annually require autologous vessel or synthetic grafts to replace coronary or peripheral arteries.<sup>31</sup> More than 500,000 coronary bypass artery graft procedures are performed annually in the United States, and peripheral disease results in more than 100,000 amputations each year.<sup>30,32</sup> The therapeutic modalities to treat cardiovascular disease are medical treatments, catheter-based interventional procedures, and surgical procedures. The medical treatments focus on disease

modification by treating conditions that lead to cardiovascular disease such as management of hypertension, hyperlipidemia, and diabetes, and cessation of smoking. The interventional procedures such as angioplasty, stents, or thrombolytic therapy offer a less invasive treatment than does surgery for patients with occlusive vessel disease. The surgical options for vascular disease are bypass surgery, endarterectomy, or open thrombectomy.

The currently available conduits for bypass procedures include autologous artery or vein, prosthetic material, and cadaveric vein. Autologous tissue provides the best outcome; however, autologous tissue in many patients may not be available or may not be suitable. Prosthetic materials, such as polytetrafluoroethylene (PTFE) and Dacron, perform well for large-diameter arteries in peripheral vascular disease. In contrast, no prosthetic material is well-suited for small-diameter (less than 6 mm) applications.<sup>33</sup> Another growing area of need for bypass procedures is hemodialysis—more than 250,000 patients require chronic hemodialysis access and frequent graft replacement.<sup>34</sup> Tissue engineering technology has the potential to generate engineered arterial grafts for multiple applications in cardiovascular surgical therapy.

## Structure and Function

A blood vessel is composed of the intima, media, and the adventitia.<sup>35</sup> The intima contains the endothelium, lamina propria, and the internal elastic lamina. The media contains the smooth

muscles arranged concentrically, elastin and collagen fibers, and the external elastic lamina. The adventitia contains connective tissue, fibroblasts, vasa vasorum, and nerve cells. Arterial blood vessels are classified as elastic arteries, muscular arteries, and arterioles. The large elastic arteries have higher elastin content and fewer smooth muscle cells relative to diameter than other vessel types. Muscular arteries contain a greater number of smooth muscle cells in proportion to their diameter, and the adventitia is composed of a thicker layer of connective tissue in comparison to the elastic arteries. The arterioles consist of a thin layer of smooth muscle and range from 40 microns to 9 microns in diameter.<sup>35</sup>

### Design Goals for a Tissue-Engineered Vessel

The primary design goal of a tissue-engineered blood vessel is to create a graft that will maintain patency in small-diameter applications, eg, 2 to 6 mm. The desirable features of the tissue-engineered vessel are a patency rate comparable to autologous vein, similar mechanical properties to a native artery or vein, minimal immunologic or infective risk, a reasonable time to produce the grafts, and cost effectiveness. The reported 5-year patency rates using autologous vein grafts for below-the-knee applications are 50% to 76%.<sup>33</sup> However, the patency rates for below-the-knee bypass with PTFE have a 2-year patency rate of 30% and only 18% after 5 years.<sup>36,37</sup> In cardiac bypass, the 10-year patency rate of autologous vein is 50%.<sup>38</sup> The current blood vessel prosthetics, Dacron and PTFE, both cause platelet and fibrin deposition that leads to thrombosis. Tissue-engineered grafts probably need some amount of lead time to culture, which could limit their clinical utility for some patients. New technology using tissue-engineered grafts will likely be more expensive than the current prosthetic materials. However, because there are no effective, synthetic, small-diameter grafts, the increase in cost for a tissue-engineered graft could be justified when considering reduced cost of chronic wound management, multiple surgical procedures with ineffective material, and reduction in patient morbidity.

### Cell Source

The predominant two cell types that form a vessel are smooth muscle cells and endothelial cells. The contribution of the live smooth muscle cells may not be critical for the function of the tissue-engineered vascular graft. Many tissue-engineered models take advantage of the process of decellularization to reduce the immunologic reaction.<sup>39</sup> The decellularized scaffold is able to maintain the tissue architecture and the extracellular matrix components to encourage repopulation of the graft with host cells.<sup>40,41</sup> The function of the endothelial cell layer on the lumen is to maintain an antithrombotic environment. Studies have shown that without an endothelial coating for a tissue-engineered small vascular graft, there is a significantly higher rate of thrombosis.<sup>41,42</sup> The options for obtaining endothelial cells

from a recipient patient are to use primary vascular endothelial cells, or to isolate progenitor endothelial cells from the marrow or the peripheral blood. Primary endothelial cells from endogenous veins have the benefit of established isolation techniques that reliably produce endothelial cell cultures. The limitations to using a primary endothelial cell source are that it requires an operation to harvest a vein, derived endothelium is venous rather than arterial, and that they will have been exposed to potential disease processes. The most common source for endothelial progenitor cells is from the peripheral blood, although there are also reports of obtaining progenitor cells from marrow. The endothelial progenitor cell can be defined as a cell that has high proliferative capacity, is capable of clonal expansion, and can differentiate into endothelial cells. The first description of endothelial progenitor cells was by Asahara and colleagues in 1997.<sup>43</sup> They demonstrated that the isolated cells from the peripheral blood mononuclear fraction that were sorted for CD34, expressed cell surface markers of endothelial cells such as vascular endothelial growth factor receptor-2 (VEGFR-2) and were positive for CD31. In addition, these progenitor cells exhibited functional properties of endothelium, by production of nitric oxide in response to acetylcholine and to VEGF.

### Scaffold

There are several approaches to developing a scaffold for tissue-engineered vessels. One approach is to use cells to form an extracellular matrix without a synthetic scaffold, while the other approach is to use synthetic polymers or natural materials to form the cell culture scaffold. In the first approach, L'Heureux and colleagues cultured sheets of fibroblasts or smooth muscle cells and wrapped the sheets around a mandrel (scaffold) in several layers to form a tubular structure.<sup>44</sup> The burst pressure, compliance, and the wall thickness were all similar to native arteries.<sup>44</sup> The biomimetic system developed by Niklason and colleagues for small-diameter vessels calls for placing aortic smooth muscle cells on a PGA scaffold under pulsatile flow for 8 weeks.<sup>42</sup> The PGA scaffold degrades, and the resulting vessel is composed primarily of smooth muscle cells and collagen. The tissue-engineered vessel does exhibit contractile properties, although at 20% that of native vessels. The mechanical properties demonstrate burst pressure and compliance similar to native vessels.<sup>42</sup> Other approaches use a collagen gel that is coated with a microporous polyurethane film.<sup>45</sup> The collagen gel is compressed to remove water, and then wrapped with the polyurethane film. The compliance of such constructs in the higher strain range is similar to native arteries.<sup>45</sup> Bruch et al created a tissue-engineered graft by mixing fibrinogen and thrombin in equal volumes into a mold with specific length and diameter.<sup>46</sup> The only mechanical characterization was burst strength, which was significantly less than native arteries.<sup>46</sup> A combination method evaluated in animal models has been to decellularize a native vessel and then to seed this with endothelial cells.<sup>41</sup> There is evidence to suggest that decellularization invokes minimal immunologic

reaction, in comparison to cryopreserved vessels, which lead to moderate immunologic reaction.<sup>47</sup> The tissue-engineered graft developed by Shin'oka et al was designed for low-pressure venous systems, using a copolymer lactide and  $\epsilon$ -caprolactone 1:1 scaffold.<sup>48</sup> The graft is seeded with marrow cells at the time of operation and then sprayed with fibrin sealant, after which the graft is implanted.

### Bioreactor Design

The two most successful bioreactor designs for tissue-engineered vessels are to wrap sheets of fibroblasts around a mandrel or to use a flow bioreactor system to culture a vessel from smooth muscle cells. The first method involves obtaining a skin biopsy to culture fibroblasts.<sup>44</sup> The fibroblasts are grown in sheets, and the first sheet is wrapped around a mandrel to form a tubular structure. The inner sheet is dehydrated to form an acellular inner membrane. Additional sheets of living cells are then placed around the inner membrane, and the tissue remains in culture to mature. The last step of this process involves obtaining endothelial cells harvested from saphenous vein to coat the luminal surface of the graft. The entire process takes approximately 28 weeks to form a vessel for implantation. The second technique is to obtain smooth muscle from a vessel biopsy, and to seed the smooth muscle on a biodegradable PGA scaffold.<sup>42</sup> The scaffold with the cells remains static for 1 week to allow for attachment, and then the construct is exposed to pulsatile flow for 7 to 8 weeks. The luminal side of the graft can be seeded with endothelial cells. This process takes around 10 weeks total to create a vessel for implantation.

### Preclinical Data

Table 61-2 lists some of the important animal models that have combined a cell source with a scaffold to produce a functional vascular graft. L'Heureux recently reported using the fibroblast

sheet scaffold and primary endothelial cells in a nude rat model and an immunosuppressed primate model.<sup>49</sup> The patency rates in both of these studies are high, and there were no graft mechanical failures. In Argentina this has been approved for a clinical study for arteriovenous graft in kidney failure patients and in England for coronary bypass graft.<sup>50</sup> These studies are ongoing and actively enrolling patients. The tissue-engineered smooth muscle grafts seeded with primary endothelial cells by Niklason (unpublished observations) also had high patency rates in a porcine carotid model and no incidence of mechanical failure. Niklason's group has demonstrated the mechanical stability of the tissue-engineered grafts after a decellularization process. The option of decellularization would allow for increased availability with the advantage of being able to create an artery and then to store the construct until needed. Other in-vivo studies have used a decellularized native vessel, and then coated the acellular construct with endothelial cells derived from peripheral blood endothelial progenitor cells.<sup>41</sup> The patency rate could be attributed to the activity of the endothelial cells because they were fluorescently labeled and remained on the graft at the time of harvest. The Matsuda group that used a collagen gel with a microporous polyurethane film combined their unique scaffold with endothelial cells that were derived from endothelial progenitor cells.<sup>48,51</sup> They also had very high patency rates in their carotid canine implant model, and the endothelial cells derived from progenitor cells provided a nonthrombotic environment. An interesting blood vessel graft for the application of large vein bypass for infants has achieved clinical success in Japan. Shin'oka has used a polylactide/caprolactone-reinforced PGA mesh coated with marrow mononuclear cells.<sup>48</sup> In the case study, they performed a cavopulmonary bypass in 23 patients and a sheet graft in 19 patients. The median follow-up was 16.7 months with a 100% patency rate and without any major complications.

**Table 61-2.** Selected Animal Models that Have Produced a Functional Vascular Graft

| Reference                        | Model                                                                            | Cell Source                                               | Scaffold                                        | Outcome                                                                |
|----------------------------------|----------------------------------------------------------------------------------|-----------------------------------------------------------|-------------------------------------------------|------------------------------------------------------------------------|
| Shin'oka <sup>48</sup>           | Human tube graft cavopulmonary connection (n = 23)<br>Sheet graft patch (n = 19) | Endothelial cells—Marrow                                  | Polylactide /caprolactone- reinforced PGA mesh  | Follow-up: median 16.7 months<br><br>Patency: 100%<br>No complications |
| L'Heureux <sup>49</sup>          | Canine aortic graft<br>Rat aortic graft (n = 27)<br>Primate aortic graft (n = 3) | Scaffold—fibroblast<br><br>Endothelial cell—primary cells | Fibroblast sheet                                | Rat: patency 85% at 90-225 days<br>Primate: all patent at 6-8 weeks    |
| Niklason <sup>42</sup>           | Porcine saphenous artery graft (n = 4)                                           | Endothelial—primary<br>SMC—porcine aortic                 | Tissue-engineered SMCs over PGA mesh            | 100% patent at 4 weeks                                                 |
| Cho <sup>52</sup>                | Canine carotid (n = 6)                                                           | Endothelial—marrow<br>SMC—marrow                          | Decellularized canine carotid                   | 100% patent for 8 weeks                                                |
| Bischoff and Mayer <sup>41</sup> | Sheep (n = 7)                                                                    | Endothelial—peripheral blood                              | Decellularized porcine iliac vessel             | 100% patent at 15 or 130 days                                          |
| Matsuda <sup>51</sup>            | Canine (n = 12)                                                                  | Endothelial—Peripheral Blood                              | Collagen mesh reinforced with polyurethane film | 11/12 patent for up to 3 months                                        |

PGA = polyglycolic acid; SMC = smooth muscle cell.

## Future of Vascular Tissue Engineering

The field of vascular tissue engineering has demonstrated proof-of-principle methods with various scaffolds and cell sources that are now ready to emerge in the clinical arena. The animal studies provide insight into the potential of the application, but further human trials are needed to determine efficacy once the safety of these devices has been verified in animals. Tissue-engineered vessels appear to be the next generation of small-diameter bypass grafts to address the growing need for alternative materials for small-caliber arterial replacement. If the tissue-engineered grafts perform close to autologous veins in terms of mechanical characteristics and nonthrombotic surface, the morbidity and mortality of cardiovascular disease could be dramatically reduced.

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## Bone Tissue Engineering

Bone tissue regeneration is a potential clinical treatment to replace loss of skeletal function for orthopedic and oral-maxillary applications. The most common needs for bone replacement materials are for trauma, tumor, infection, and inborn skeletal disorders. The current bone replacement materials are derived from autologous bone, allogeneic bone, demineralized bone matrices, and synthetic biomaterials. The first choice for bone repair has been the autologous bone graft.<sup>53,54</sup> The advantages of autologous tissue are a lack of an immune reaction and the provision of bone cells for osteogenesis. In addition to the direct osteogenic effects, the autologous bone graft can stimulate recruitment of other mesenchymal cells through stimulation by bone morphogenic proteins.<sup>54</sup> However, the use of autologous bone grafting is limited by the morbidity associated with the donor site. The usual sites to harvest bone grafts are the iliac crest and the fibula. After the procedure, up to 20% of patients have significant pain at the donor site.<sup>55</sup> In a large review of iliac crest donor sites, major complications included vascular injuries, deep infections, neurologic injuries, and deep hematoma, with a combined incidence of 6%.<sup>56</sup> An alternative to autologous bone grafting is allogeneic bone grafts. The mechanical properties of processed allogeneic bone tissue are similar to autologous bone, although the ability to induce bone healing is reduced.<sup>53</sup> For smaller defects, such as bone chips in a distal radial fracture or for use in a cervical fusion, allogeneic bone seems to be comparable to autologous bone.<sup>57,58</sup> Allogeneic bone groups had a longer time to fusion in cervical fusion, but this did not affect the fusion rate or collapse rate.<sup>58</sup>

The acrylic bone cements are a biomaterial that has been introduced for fixation of hip prostheses and to repair craniofacial defects. They provide mechanical support, but do not integrate into the bone and are minimally reabsorbed. This results in long-term failure at the interface between the bone and the prosthetic, which has led to metal prostheses coated with hydroxyapatite that do not use bone cement. The cementless hydroxyapatite-coated prosthesis is under investigation to improve osseointegration and prevent bone remodeling that

leads to long-term failure.<sup>59</sup> Another new material for craniofacial defects is a hydroxyapatite-based paste. The paste is readily available, can be easily molded into shape, demonstrates osteoconduction, has good mechanical strength, lacks immune response or risk of viral transmission, and has the potential for bone replacement.<sup>60</sup>

## Structure and Function

Bones are composed of extracellular bone matrix and bone cells that form the shape of long, short, flat, or irregular bones.<sup>35</sup> The bone matrix consists of an organic component of collagen and proteoglycans. The inorganic component of the bone matrix is a calcium phosphate crystal called hydroxyapatite. The bone cells can be classified as osteoblasts, osteoclasts, and osteocytes. Osteoblasts are bone cells that produce collagen, proteoglycans, and form bone. The osteoclasts are the cells responsible for the breakdown of bone by producing an acidic environment that causes decalcification of the bone matrix. The osteocytes are osteoblasts that are surrounded by matrix. The interaction of bone cells with hormones regulates the homeostasis of calcium and phosphate in the blood.

## Scaffold

The structure of the scaffold for bone regeneration is related to the function of bone. A bone scaffold should provide means of cell migration and proliferation, generate appropriate mechanical strength, integrate into the native bone matrix, stimulate vascularization, slowly degrade with increasing cellular infiltration, and not elicit an immunologic reaction. For example, the different requirements for the strength of long bones and for the flexibility of craniofacial bones may lead to the development of different scaffolds for these applications. The matrices used for bone tissue engineering can be classified as calcium phosphate derivatives or as synthetics. Hydroxyapatite is a calcium phosphate compound that is the primary component of bone. Hydroxyapatite as a scaffold offers a high degree of biocompatibility, the osteoconductive potential of hydroxyapatite, and slow degradation. Hydroxyapatite is a porous material that will allow ingrowth of bone and vascular cells.<sup>61</sup> However, hydroxyapatite implants are known to be brittle and lack pliability to conform to irregular forms.<sup>61</sup>

Hydroxyapatite cements have been approved for clinical use for non-stress-bearing cranial defects. The clinical experience with one of the hydroxyapatite bone cements in 100 patients over a minimum of 2 years demonstrated a success rate of 97% and a low rate of infection.<sup>62</sup> Hydroxyapatite cements can be used for craniofacial repairs because they integrate into the bone and can be easily shaped to fit unique three-dimensional structures.<sup>62</sup> The properties of calcium phosphate or hydroxyapatite grafts can be altered by integrating other biomaterials to form a composite material. The composites that investigators have evaluated include calcium phosphate-chitosan, calcium phosphate-poly(lactic acid), and hydroxyapatite-collagen scaffolds. The purpose for fabricating composite calcium phosphate is to

improve the biodegradability and the bioactivity. Pore size and porosity are important factors in bioactivity, affecting cell adhesion, cell growth, the exchange of nutrients and waste products, and vascularization. A calcium phosphate-chitosan composite has shown improved strength characteristics in comparison to calcium phosphate cement alone.<sup>63,64</sup> The fast-setting composite material is prepared by mixing a calcium phosphate-mannitol powder with chitosan water to form a paste that can be molded into shape or injected.<sup>63</sup> A composite material of collagen and hydroxyapatite has been shown to support bone cell growth and act as a biocompatible material *in vivo*.<sup>65,66</sup> There are different processing techniques to produce collagen-hydroxyapatite composites, such as gels, films, coatings, and hydroxyapatite-collagen matrices. The hydroxyapatite-collagen material creates a favorable environment for cell attachment and growth, and the cells are functional as assessed by the deposition of new bone.<sup>66</sup>

### Vascularization

A vascular supply for a tissue-engineered bone graft is necessary for bone growth and survival. The two approaches to vascularize a bone graft are to rely on angiogenesis of the donor, or to form a vascular network within the graft before implantation. The current bone graft substitutes depend on vascularization through angiogenesis from the donor after implantation of the scaffold with bone cells. An explanation for the success of bone tissue engineering in rodents in comparison to larger animal models is that the marrow-seeded grafts in rodents are small enough to allow for diffusion of oxygen and nutrients to allow cell survival before angiogenesis and new vessel formation.<sup>67</sup> One method to improve vascularization is to stimulate angiogenesis on the graft. This may be accomplished by adding a combination of growth factors such as VEGF, platelet-derived growth factor-BB, and transforming growth factor- $\beta$  (TGF- $\beta$ ).<sup>68,69</sup> In a rat cranium bone tissue engineering application, the addition of VEGF was shown to improve vascularization and bone mineralization.<sup>70</sup> However, it is not clear if the addition of the growth factors will vascularize a bone graft in a short enough time to maintain cell viability in a larger graft, such as that required for patients.

The second method would be to vascularize the graft before implantation by heterotopic bone induction or to form a vascular network within the graft *in vitro*. Warnke and colleagues reported a successful extended mandible reconstruction by growth of a custom bone transplant from the latissimus dorsi muscle of an adult male patient.<sup>71</sup> The custom bone transplant was prepared with a titanium mesh that was coated with recombinant bone morphogenic protein 7 (BMP-7) and seeded with marrow aspirate. The bone construct was implanted into the latissimus dorsi muscle for 7 weeks, then was transplanted as a free bone-muscle flap to repair the mandibular defect. A postoperative computed tomography (CT) scan demonstrated new bone formation, and a nuclear scan showed bone remodeling and mineralization inside the mandibular transplant. *In vitro*, bone tissue engineering strategies could utilize a co-culture system to

form a vascular network using endothelial cells in a matrix that is later seeded with marrow cells.

### Growth Factors

Bone morphogenic proteins are osteoinductive factors that can be used to coat scaffolds. BMPs are glycoproteins that belong to the TGF- $\beta$  family and bind to extracellular matrix components. BMPs are the biologically active component in demineralized bone matrix that leads to formation of new bone.<sup>72</sup> BMP-2 and BMP-7 are involved in bone formation and osteoblast differentiation. These recombinant proteins have been used for applications such as spinal fusions, internal fixation of fractures, treatment of bone defects, and reconstruction of maxillofacial conditions. BMPs stimulate bone repair through recruitment, proliferation, and differentiation of MSCs.<sup>73</sup> Preclinical studies have demonstrated the efficacy of BMPs in healing a variety of bone defects in many different animal models. The bone formation and healing of long bone defects with recombinant BMP-2 and collagen scaffolds has been demonstrated in rats, rabbits, and dogs.<sup>74-76</sup> Similar results have been shown with BMP-7 and collagen scaffolds in healing long bone defects in multiple animal species.<sup>77,78</sup>

Two recombinant BMP devices have been approved by the FDA. OP-1 (Stryker Biotech, Hopkinton, MA) has been approved for spinal fusion procedures under the humanitarian device exemption process. The OP-1 device is recombinant BMP-7 and bovine collagen combination that is used to form a paste.<sup>79</sup> The other device is the Infuse Bone Graft/LT-CAGE (Medtronic, Minneapolis, MN).<sup>79</sup> The indication for the device is for lumbar spinal fusion to treat degenerative disc disease. The device consists of a metallic spinal fusion cage with rh-BMP-2 on a bovine collagen scaffold inside the cage.

### Cell Source

The options for osteogenic cells in bone engineering are osteoblasts, periosteal cells, and MSCs. Osteoblasts and periosteal cells can be isolated from a biopsy of the calvarium, periosteum, or trabecular bone.<sup>80,81</sup> Osteoblasts and periosteal cells are autologous, primary cell sources for bone engineering. Mesenchymal stem cells are utilized for bone regeneration, because the cells have a large proliferative potential, can be differentiated down osteogenic lineage, and can be harvested by a less invasive marrow aspiration procedure. The most common cell source for bone tissue engineering applications is currently MSCs.<sup>82</sup>

Mesenchymal stem cell differentiation to osteogenic cells was first described by Friedenstein.<sup>83</sup> Mesenchymal stem cells have been induced to differentiate into osteoblasts, chondrocytes, adipocytes, and myoblasts.<sup>84</sup> Caplan was the first to take advantage of the potential of MSCs by seeding the cells onto a porous calcium phosphate ceramic.<sup>85</sup> In a subcutaneous *in-vivo* rat model, they demonstrated osteogenesis with an early phase resulting from the implanted mesenchymal cells and a late bone growth phase resulting from the host cells. Since these early papers, there have been many studies on the use of MSCs for bone tissue



engineering applications. The use of MSCs on biomaterial scaffolds has accelerated bone formation and bone integration in vivo. Mesenchymal stem cells improved osteogenic potential directly by differentiating into osteoblasts, and indirectly by releasing bone growth factors that enhance migration and differentiation of endogenous osteogenic cells.<sup>86</sup>

Preclinical and Clinical Studies

The evaluation of new cell-based therapies for bone repair are predominantly in the preclinical stages, with only a few human case reports. There are many studies optimizing scaffolds and evaluating the function of the MSCs. A limited number of groups are studying the challenging task of using a biomaterial in combination with a cell source to repair large osseous defects in animal models. All of the studies in Table 61-3 demonstrate that an MSC-seeded scaffold produces significantly greater bone formation than cell-free grafts, and in most of the studies the MSC-seeded scaffolds resulted in similar bone formation as bone grafts. In addition to the use of calcium phosphate as the scaffold, investigators have also used coral. The rationale for coral as a scaffold is the porous structure and more rapid degradation of coral in comparison to calcium-phosphate-based materials.<sup>87</sup> The more rapid degradation time of the coral may reduce the inflammatory response and allow for growth of new bone tissue more quickly.<sup>88,89</sup> The number of cells seeded may be an important variable for bone growth in the implanted graft. High cell density at the time of seeding may have a negative impact on the graft by not allowing the cells to spread out on the graft and proliferate. Another consequence of high cell density without adequate vascularization is the formation of a hypoxic environment in the center of the graft.<sup>87,89</sup>

Case reports have described bone tissue engineering in clinical practice. Vacanti et al reconstructed a distal thumb avulsion with periosteal cells and a coral scaffold.<sup>93</sup> At 3 months the patient was able to return to work, and at 28 months the thumb had normal length and strength. In a separate study, three patients with large bone defects in the extremities were treated

with marrow cells and macroporous hydroxyapatite scaffold.<sup>94</sup> All three patients recovered limb function. Thus, in the appropriately selected patients, it appears that bone tissue engineering concepts can be applied to repair bone defects in humans.

Conclusion

Bone tissue engineering has the potential to provide better treatment options for bone reconstruction in orthopedic, craniofacial, and plastic surgery. Bone tissue engineering may provide a material that will integrate into native bone for a complete functional recovery, without the morbidity associated with autologous donor bone harvest. The integration of marrow MSCs with an optimized scaffold that includes some combination of BMPs will enhance the local environment for bone growth and remodeling by the native tissue. The most efficient technique to vascularize large bone grafts is an important area of research that is needed to improve bone graft survival. The challenges remain to create products that address the wide spectrum of clinical needs, from pliable materials for facial reconstruction to the strength required for long bone reconstruction.

Cartilage Tissue Engineering

The motivation for cartilage tissue engineering is to improve treatments for cartilage degeneration that is caused by disease, trauma, or congenital abnormalities. Some examples of cartilage disorders are osteoarthritis, cartilage injuries, herniated disc, costochondritis, and achondroplasia. Osteoarthritis is the most common cause of arthritis and the leading cause of disability that primarily affects middle-aged and elderly population.<sup>95</sup> The incidence of osteoarthritis in people older than 75 is greater than 80%, and radiologic evidence in those older than 50 is greater than 50%.<sup>96</sup> As the population in the United States continues to age, the demand for treatments that allow patients to maintain an active lifestyle will increase. The surgical options are arthroscopic surgery or joint replacement, although the efficacy of

Table 61-3. Selected Animal Models that Have Produced Bone Formations

| Reference                 | Model                                    | Cell Source                   | Scaffold                                           | Outcome                                                                                                                     |
|---------------------------|------------------------------------------|-------------------------------|----------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------|
| Schliephake <sup>90</sup> | Sheep mandible defects 35-mm length      | Marrow cells                  | Heat-treated bovine bone, porous calcium phosphate | Greater bone formation in seeded scaffold at 5 months                                                                       |
| Shang <sup>91</sup>       | Sheep cranial bone defect 20-mm diameter | Mesenchymal stem cells (MSCs) | Calcium alginate                                   | At 18 weeks scaffold and MSCs almost healed vs scaffold with open defect                                                    |
| Arinze <sup>92</sup>      | Canine femur defect 21-mm length         | Allogeneic MSCs               | Hydroxyapatite-tricalcium phosphate                | No adverse host response; greater amounts of bone vs cell-free implants                                                     |
| Zhu <sup>87</sup>         | Goat femur defect 25-mm length           | MSCs                          | Coral                                              | Cell seeded group at 4 months radiographic union; structural properties similar native bone; cell-free group no bone growth |
| Hou <sup>88</sup>         | Rabbit cranial defect 15-mm diameter     | MSCs                          | rhBMP-2/coral                                      | At 16 weeks by radiography MSC/rhBMP-2/coral similar to iliac autograft and greater than cell-free rhBMP-2/coral            |
| Viateau <sup>89</sup>     | Sheep metatarsal defect 25-mm length     | MSCs                          | Coral                                              | MSCs and autograft similar bone formation, much greater than cell-free scaffold                                             |

arthroscopy with debridement or joint irrigation of the knee has been recently challenged.<sup>97</sup> Joint replacement surgery is indicated when the patient no longer responds to nonpharmacologic and drug treatments.

### Structure and Function of Cartilage

The structure of cartilage is composed of a dense extracellular matrix with embedded chondrocytes.<sup>35</sup> The extracellular matrix includes collagen Type II fibers, elastin fibers, and proteoglycans that give cartilage viscoelastic and mechanical properties. Cartilage is avascular and relies on diffusion for nutrients, oxygenation, and removal of waste. The three types of cartilage are hyaline, elastic, and fibrocartilage. Hyaline cartilage is the most common form of cartilage, and consists primarily of Type II collagen and proteoglycans. Hyaline cartilage forms the skeleton of the embryo, which is later converted to bone as the embryo matures. In adults, hyaline cartilage can be found in the trachea, larynx, nose, bronchi, load-bearing joints, and the ends of the ribs. Elastic cartilage contains collagen and elastin fibers; it is found in the pinna of the ear and forms the epiglottis. Fibrocartilage contains more collagen than hyaline, and is found in areas that require great tensile strength. Fibrocartilage is located between intervertebral discs, between the articulations of the hip, and at segments connecting tendons or ligaments to bone. The function of cartilage is to reduce friction of articulating bones and to assist with load bearing.<sup>35</sup>

### Cell-Based Therapies

One cell-based treatment for the repair of cartilaginous lesions is autologous chondrocyte implantations. A first procedure requires the harvesting of cartilage from non-load-bearing region of bone. The tissue is cultured and chondrocytes are isolated and expanded. In a second procedure, the lesion is surgically debrided and the chondrocytes are implanted under a periosteal flap. However, the conclusion of a recent systematic review of three trials and nine case reports revealed there was not sufficient evidence that autologous chondrocyte implantation is more effective than alternative therapies for chondral lesions of the knee.<sup>98</sup> Two other reviews of available evidence by the National Institute of Clinical Excellence and Blue Cross and Blue Shield Association concluded that autologous chondrocyte implantation is not recommended over alternative treatments.<sup>99,100</sup>

### Cell Source

The cell sources for cartilage are harvested primary chondrocytes or MSCs that are induced to differentiate into chondrocytes. Primary chondrocytes can be harvested from articular, septal, auricular, and costal cartilage. Studies have reported different findings on the characteristics of cartilage harvested from various sites in animal models.<sup>101-103</sup> Human nasal chondrocytes proliferate faster than articular chondrocytes, possess a higher chondrogenic potential, and survived 6 weeks implantation into nude mice. Hence, nasal cartilage may be a better cell source than articular cartilage for tissue engineering applications.<sup>101</sup>

Primary chondrocytes can be stimulated to proliferate and differentiate by various factors. Chondrocytes dedifferentiate during in-vitro expansion in monolayer culture, taking on a fibroblast-like morphology and producing macromolecules such as collagen Type I and versican.<sup>104</sup> Chondrocytes can be induced to redifferentiate by the addition of growth factors, forming pellets at a high cell density, and by growth in a three-dimensional scaffold.<sup>105,106</sup> The addition of specific growth factors, such as TGF $\beta$ -1 and basic fibroblast growth factor (FGF), to monolayer cultures has been shown to induce dedifferentiation and enhance the proliferative capacity of the cells in animals and in humans.<sup>106,107</sup>

Many growth factors and combinations of growth factors have been investigated to optimize MSC differentiation into chondrocytes. Basic FGF (FGF-2) has been identified as the most potent mitogen in the FGF family to promote chondrogenesis and proliferation of MSCs.<sup>108</sup> Mesenchymal stem cells induced with FGF-2 in pellets differentiated to chondrocytes in rabbit, dog, and human species.<sup>109</sup> The formation of cartilage from MSCs was superior with FGF-2 in comparison to other growth factors, such as insulin-like growth factor, epidermal growth factor, and platelet-derived growth factor.<sup>110</sup> BMP-2 is found in the mesenchyme of the developing limb and is known to stimulate cartilage formation in culture. Human MSCs in the presence of BMP-2 in a serum-free pellet culture system resulted in chondrogenic lineage development only. Cells from osteogenic or adipogenic lineage were not stimulated with BMP-2. The addition of BMP-2 to mesenchymal pellet culture system appeared to produce cartilage with higher amounts of proteoglycans in comparison to BMP-4 or BMP-6.<sup>111</sup>

### Scaffold

Scaffold materials for cartilage tissue engineering are collagen, hyaluronan, fibrin sealant, and synthetic polymers. Collagen is a good biomaterial for cartilage tissue engineering applications, because it is biocompatible, biodegradable, can be molded into specific forms, and can maintain chondrocyte phenotype vis-à-vis glycosaminoglycan production.<sup>112</sup> In animal models, collagen-based scaffolds with chondrocytes have resulted in better healing in osteochondral injury models than other types of scaffolds. One method to reduce the dedifferentiation of chondrocytes with collagen is to add hyaluronic acid. Hyaluronic acid is a proteoglycan that acts to lubricate joint surfaces and assists cartilage to withstand compressive forces.<sup>113</sup> Hyaluronan must be modified by esterification or other means for structural purposes and to optimize solubility, hydration, and degradation.<sup>114</sup>

A fibrin matrix can also support the growth of chondrocytes, and an in-vivo model demonstrated regeneration of cartilage with articular cartilage defects using a fibrin system.<sup>115</sup> The preparation of fibrin needs to result in a stable gel that can support the growth of chondrocytes, otherwise fibrin gels have been known to shrink in size and degrade in a very short time.<sup>115</sup> Fibrin gels have been produced that are stable with good mechanical strength, and in-vitro cultures have shown

chondrocytes proliferate in these gels with the production of glycosaminoglycans and collagen Type II.<sup>115</sup>

The synthetic polymers PLA and PGA were investigated as cartilage scaffolds early in the study of cartilage tissue engineering. In-vitro studies demonstrated the biocompatibility of PGA with chondrocytes, and early rabbit model studies demonstrated a better healing response of PGA seeded with chondrocytes over PGA scaffold alone.<sup>116</sup>

PGA-seeded scaffolds have resulted in the greatest production of glycosaminoglycans by chondrocytes at 20 days in comparison to alginate, fibrin, and collagen.<sup>117</sup> A concern with the application of PLA/PGA polymers is an inflammatory reaction that develops after hydrolysis of the polymer. However, a laryngotracheal rabbit model with a PLA/PGA copolymer had appropriate clinical function of the implants at the time of harvest at 12 months. The scaffolds were completely resorbed without signs of a chronic inflammatory response.<sup>118</sup> Despite the success in animal models, no PLA/PGA-based materials have progressed to clinical trials.

### Bioreactor Culture System with Mechanical Stimulation

The parameters that have the greatest impact on the production of cartilage in a bioreactor are cell seeding density, mass transfer as modulated by shear stress, and compressive forces.<sup>119</sup> The seeding density of chondrocytes must be balanced by maximizing cell number to produce more extracellular matrix, without using too high of a cell number that limits mass transfer. Limited mass transfer can result in non-uniform cell distribution with higher cell numbers at the surface of the scaffold. The methods to apply shear stress and enhance mass transfer are mechanically stirring the bioreactor, direct perfusion of medium through scaffolds, and a rotating-wall bioreactor that imparts low shears and stirring.<sup>119</sup> Besides the desirable increased stimulation of extracellular matrix, the stirred mechanical systems also produce a more even distribution of cells over the scaffold. A direct perfusion system is a flow system where a cell suspension or media alone directly passes through the pores of the three-dimensional scaffold. Experiments using a direct perfusion culture system demonstrated an increase in the DNA content and an increase in glycosaminoglycans over static culture conditions.<sup>120</sup> However, the tissue-engineered perfused cartilage still has significantly less collagen in comparison to native cartilage.

Compressive forces stimulate differentiation of MSCs to chondrocytes and contribute to structural maintenance of articular cartilage in vivo. The two types of forces that in-vitro systems use are hydrostatic force and compressive force. In vivo, hydrostatic pressure is produced on the chondrocytes by the synovial fluid transmitting pressure to the water contained in the cartilage matrix.<sup>119</sup> Physiologic levels of hydrostatic pressure are in the range of 7 to 10 MPa, and because cartilage is incompressible in this range, only minimal tissue deformation occurs.<sup>121,122</sup> The duration of hydrostatic pressure, total time of application, and magnitude are variables that need to be optimized for cartilage tissue engineering applications. The most common ranges of loads are 0.1 to 15 MPa and the ranges for frequencies are

0.05 to 1 Hz.<sup>119,123,124</sup> Studies have shown that chondrocytes in an agarose scaffold with a strain of 3% to 10% at a frequency of 1 Hz and a culture time of 4 to 6 weeks have an increase in the amount of proteoglycan and collagen in comparison to static cultures.<sup>125,126</sup> In another study by Waldman, bovine chondrocytes were seeded onto a calcium polyphosphate scaffold with 5% compression at 400 cycles per day at a frequency of 0.5 Hz.<sup>127</sup> The scaffold and cells were stimulated every other day for 1 to 4 weeks, with the addition of either 2% shear or 5% shear. The combined 5% compression and 5% shear increased collagen synthesis by 76% and proteoglycan synthesis by 73% over the static controls. The authors noted that greater responses to uniaxial loading, shear, or compression alone, were observed in prior studies.

### Clinical Applications

Tissue engineering of articular cartilage could provide an alternative cell-based therapy to autologous chondrocyte implantation. Because autologous chondrocyte implantation has not shown significant improvements in patients with chondral lesions, other therapeutic options are needed for treatment of these lesions.<sup>98</sup> The only tissue engineered cartilage graft is Hyalograft C (Fidia Advanced Biopolymers, Abano Terme, Italy), which is not FDA-approved but is available in Europe. Hyalograft C is composed of autologous chondrocytes grown on a three-dimensional hyaluronic-based scaffold called HYAFF 11. The scaffold is an esterified derivative of hyaluronic acid that is biodegradable and biocompatible.<sup>128</sup> In a recent multicenter study from Italy, autologous chondrocytes were isolated from a cartilage biopsy harvested arthroscopically from a non-weight-bearing area in the knee. The cells were expanded in vitro and then cultured on the HYAFF 11 scaffold for 14 days. The engineered graft was implanted at the lesion site by a mini-arthrotomy under regional or general anesthesia. In 55 of the 141 patients, a biopsy of the implanted cartilage was taken and evaluated using the International Cartilage Repair Society visual scoring system. At a mean follow-up time of 38 months after the procedure, 71.4% of the patients stated they could do everything or nearly everything that they wanted to do with their joint compared to 4.3% before the surgery. The visual scoring of the repaired tissue at the postsurgery arthroscopy revealed normal or near normal scores in 53 of 55 patients after a mean implantation time of 14.1 months. There were 10 graft failures, which was 5.2% of the study group. The authors stated a limitation of the study was in the design of an observational trial instead of a randomized controlled trial. In a follow-up study to further characterize the nature of the biopsied tissue, biochemical analyses were applied.<sup>128</sup> The average follow-up time from grafting to biopsy was 16 months, with a range of 6 to 30 months. Ten of the 23 cases demonstrated regenerated hyaline cartilage, but a larger sample size is needed to make more definite assessments.

### Future Challenges

The challenge of cartilage tissue engineering remains to develop a therapeutic treatment for cartilage disease or injury. The

success of Hyalograft C in improving clinical symptoms is encouraging, but further evaluation of the mechanical properties and the long-term graft efficacy needs to be undertaken. A reliable cell source has been obtained by primary cell harvest, and MSCs offer an even better potential. By selecting the appropriate cell density, growth factors, mechanical stimulation, and scaffold, tissue-engineered cartilage can be successfully designed for clinical applications.

## Urology Tissue Engineering

The urinary system is exposed to a variety of insults that require reconstruction as a treatment. Frequent reasons for reconstruction in the urinary system include congenital abnormalities, cancer, trauma, infection, and iatrogenic injuries. The primary option for bladder and urethral reconstruction involves the use of gastrointestinal tissue. One congenital anomaly that may require the need for bladder reconstruction is exstrophy of the bladder. The prevalence of bladder exstrophy is 3.3 births per 100,000.<sup>129</sup> The initial treatment for this bladder defect is early primary closure. However, in some cases the bladder is too small for closure or the bladder does not develop to maintain continence. In these cases, tissue harvested from the small bowel or colon is used to augment the size of the bladder.<sup>129</sup>

Cancer of the urinary bladder is the fourth most common cause of cancer in men, and the ninth most common in women.<sup>129</sup> Invasive bladder cancer is treated by radical cystectomy with urinary diversion. The use of selective bladder preservation or partial cystectomy is inferior to radical cystectomy for invasive cancer, meaning that total bladder replacement could significantly enhance quality of life for these patients.<sup>129</sup> Blunt trauma is another cause of urinary tract injury, often associated with pelvic fractures or straddle injuries for the urethra. In the case of large defects that require diversion of the urinary system, use of gastrointestinal tract tissue is the only option.

Bowel segments that are mobilized for bladder reconstructions are associated with various complications. Complications include mucus production, chronic bacteruria, stone formation, rupture, electrolyte imbalance, and metabolic acidosis with a range of incidence from 5% to 44%.<sup>130,131</sup>

### Design Goals for Urinary Tissue

The objective of urinary tissue engineering is to provide an alternative source of tissue that is more similar to native bladder or urethra. The requirements for bladder tissue engineering are to act as a reservoir for urine, contract until empty, integrate with sites of anastomosis to prevent leakage, have mechanical strength to prevent rupture, and not cause excessive stone formation. The structure of the bladder is a hollow muscular organ that functions as a reservoir to hold urine until time of excretion through the urethra. The bladder is composed of transitional epithelium, lamina propria, muscular layer, and an adventitia. The transitional epithelium forms a layer of four to five cells when the

bladder is empty, and two to three layers when the bladder is distended.<sup>35</sup> The viscoelastic properties of the bladder are derived from the smooth muscle, the collagen content of approximately 50%, and elastin content of approximately 2%.<sup>129</sup> The bladder has a physiologic compliance that contains neural input to set the volume for micturition.<sup>129</sup>

### Cell Source

The most common cell source for bladder tissue engineering is a primary cell source from urothelial cells and visceral smooth muscle cells. Primary urothelial cells are autologous and have been demonstrated to expand the cells from a surface area of 1 cm<sup>2</sup> to 4202 cm<sup>2</sup> over a period of 8 weeks.<sup>132</sup> Phenotype and functional characteristics of harvested human visceral smooth muscle cells from normal, exstrophic, and neuropathic bladders have been evaluated. No differences between the normal and diseased bladder tissue with respect to phenotype and degree of contractility were observed.<sup>133</sup> An alternative potential cell source is marrow stem cells or embryonic stem cells that have not been exposed to fibrotic or malignant changes. Because marrow cells can differentiate into smooth muscle, investigators have evaluated if the smooth muscle cells have a similar phenotype and function if placed on a scaffold for bladder applications. One group reported bladder smooth muscle and marrow-derived smooth muscle had similar expression of actin and contractile response to calcium ionophore.<sup>134</sup> An in-vivo study in a hemicystectomy dog model with bladder cell-seeded or marrow cell-seeded small intestinal submucosa graft had similar outcomes in both grafts. After 10 weeks, the two implants had similar levels of alpha-actin with evidence of smooth muscle regeneration and muscle bundle formation.

### Scaffold

The main scaffold types for urologic tissue engineering are synthetic polymers and natural materials derived from acellular tissue matrices. It has been demonstrated that the adherence and proliferation of bladder smooth muscle cells appears to be similar across synthetic polymers, eg, poly(lactic-co-glycolic) acid (PLGA) and PCL.<sup>135</sup> Hybrid materials, such as a PLGA mesh-collagen gel, have also been shown to support the growth of urothelial and smooth muscle cells. The purpose of using a hybrid scaffold is to take advantage of the mechanical strength of the synthetic polymers and the cell adhesion properties of the collagen gel.<sup>136</sup> Tissues that are used to create an acellular matrix for bladder scaffolds are small intestine submucosa, native bladder tissue, and bladder submucosa.<sup>137,138</sup> Bladder replacement grafts composed of small intestine submucosa have demonstrated regeneration of normal bladder with urothelium, smooth muscle, and serosal layers in canine and rat models.<sup>138</sup>

### Preclinical and Clinical Trials

The tissue-engineered bladder has progressed from in-vitro models to a clinical trial. The canine and rabbit models have demonstrated the success in creating an autologous neo-bladder

in vivo. Table 61-4 lists some of these studies that describe the cell source, scaffold, and the outcome. The Atala group has successfully proliferated the bladder urothelial and smooth muscle cells from a small biopsy, and used a cell-free bladder construct as the scaffold. They demonstrated in a canine 50% cystectomy model that the cell-seeded graft had a 99% increase in the capacity of the bladder.<sup>137</sup> Histologic analysis confirmed the presence of nerve fibers in the tissue-engineered bladders. The Atala group has also been applying the same method for urethral replacement.<sup>139</sup> In the study by Kropp in 2004, well-expanded bladders were generated with acellular porcine small intestine submucosa (SIS) in a canine cystectomy model using cell-free grafts.<sup>138</sup> A later study by Kropp revealed improved results with bladder cell-seeded SIS or marrow-derived smooth muscle cell-seeded SIS in comparison to the control group of cell-free scaffolds.<sup>134</sup> In a third study of a canine bladder replacement cystectomy of 90%, the cell-seeded and cell-free scaffolds produced similar results of poor bladder regeneration.<sup>140</sup> The authors attribute the lack of bladder regeneration to a poor vascular network and increased inflammation produced by the large implants. The viability of the implanted graft depends on vascular ingrowth from the surrounding tissues, and the vascular network may not have been able to develop at a rapid enough rate to support such a large tissue graft. The increased inflammation may have been a result of the technique to create the large defect.

In a recent publication, the successful techniques for bladder regeneration in animal models were translated to a human trial.<sup>141</sup> The study identified seven patients with a primary diagnosis of myelomeningocele with poorly compliant bladders. The age range of the patients was from 4 to 19 with a mean age of 11 years. The cell source was from a bladder biopsy, and the scaffold was made from collagen or from a composite of collagen and polyglycolic acid. The scaffold for the first four patients was made of decellularized bladder submucosa. The last three

patients used the composite material that was sized for each individual based on age-appropriate bladder size and dimensions obtained from CT scan. The length of time between the bladder biopsy and the tissue-engineered bladder implantation was approximately 7 to 8 weeks. The patients were followed for 22 to 61 months with a median time of 46 months. The postoperative urodynamic studies with the tissue-engineered bladders showed varying degrees of contractility, capacity, and compliance. The volume and the compliance increased postoperatively, with the most significant improvement in the composite tissue-engineered scaffold that was implanted with an omental wrap. The results of the urodynamic studies were similar to those of gastrointestinal segment augmentations, and the patients did not develop any of the complications associated with the gastrointestinal segment augmentation. The authors commented that they added the composite material to the study because the PGA improved the structural integrity of the tissue while the collagen continued to support cell growth. The positive preliminary data from this trial led to a Phase II trial to provide more data on the efficacy of the tissue-engineered approach to bladder augmentation.

### Future Challenges

The use of tissue-engineered bladders in a clinical application demonstrates the exciting opportunities in the field of urothelial tissue engineering. Additional improvements in the existing approach may be to use marrow stem cells as a smooth muscle cell source, providing a vascular network before implantation, stimulation of neuronal integration into the graft, and continued optimization of the scaffold. Although there is evidence to suggest that viable bladder cells can still be harvested from diseased tissue, if bladder smooth muscle cell can be derived from marrow stem cells it may be a more attractive alternative cell source. Another potential clinical application is bladder reconstruction

**Table 61-4.** Selected Animal Models that Have Produced Autologous Neo-Bladders

| Reference                      | Model                                                                 | Cell Source                                       | Scaffold                            | Outcome                                                                                           |
|--------------------------------|-----------------------------------------------------------------------|---------------------------------------------------|-------------------------------------|---------------------------------------------------------------------------------------------------|
| Yoo/Atala <sup>137</sup>       | Canine 50% cystectomy for 2 and 3 months (n = 10)                     | Bladder urothelial and smooth muscle              | Canine allogeneic bladder submucosa | Cell-seeded with 99% increase in capacity; cell-free only 30% increase                            |
| DeFilippo/Atala <sup>139</sup> | Rabbit urethral 1-cm segment resection 1, 2, 3, and 6 months (n = 24) | Bladder urothelial and smooth muscle              | Porcine bladder submucosa           | Cell-seeded graft normal structure 1 month; cell-free strictures/collapse at all timepoints       |
| Kropp/Zhang <sup>138</sup>     | Canine 40% cystectomy for 10 weeks (n = 6)                            | None                                              | Small intestine submucosa           | Distal SIS bladders well expanded                                                                 |
| Zhang/Kropp <sup>134</sup>     | Canine 40%-50% cystectomy for 10 weeks (n = 6)                        | Bladder urothelial and SMC<br>Marrow-derived SMCs | Small intestine submucosa           | Both bladder and MSC-seeded grafts with smooth muscle; cell-free with smooth muscle only at edges |
| Zhang/Kropp <sup>140</sup>     | Canine 90% cystectomy for 1, 5, 9 months (n = 22)                     | Bladder urothelial and SMC                        | Small intestine submucosa           | Limited bladder regeneration in cell-seeded and cell-free group                                   |

SIS = small intestine submucosa; SMC = smooth muscle cell; MSC = marrow stem cell.

after cancer resection, utilizing harvested cells from an area of bladder without any neoplastic changes. The application of bladder tissue engineering for large cystectomies may require a vascular network before implantation, as suggested by data from the canine model.<sup>140</sup> The neuronal integration of the graft is important to restore the natural voiding mechanism. The clinical data demonstrated in myelomeningocele patients that the urodynamics and the dry intervals between straight catheterizations improved postoperatively.<sup>141</sup> Optimization of the scaffold for more desirable properties such as improved mechanical characteristics and improved control over degradation in response to tissue remodeling are two key issues. As tissue-engineered products progress from case studies to commercial applications, process methods to reduce the potential of infection and to achieve high-quality standards will be important issues for these new medical devices.

## Cardiac Tissue Engineering

Cardiovascular disease is the leading cause of mortality in the United States. The number of people in the United States with one or more types of cardiovascular disease is approximately 1 in 3.<sup>30</sup> Patients who survive a myocardial infarction experience myocardial damage that can eventually result in congestive heart failure. Other causes of heart failure include hypertension, cardiomyopathy, valvular disease, and cardiac arrhythmias. The number of deaths related to heart failure increased 28% in the period of 1994 to 2004.<sup>142</sup> The mortality data for heart failure is comparable to that of cancer. The pathophysiology of heart failure is the inability of the heart to maintain adequate cardiac output because of injury to the cardiac myocytes that causes an increase in wall stress and forward pumping failure.<sup>143</sup>

The treatment options for severe heart failure range from pharmacologic therapy to heart transplant. The nonpharmacologic treatments include cardiac resynchronization, coronary revascularization, left ventricular assist device implantation, and heart transplantation. The number of people requiring heart transplantation continues to increase, but the number of people receiving heart transplants has been on the decline over the past 10 years.<sup>144</sup> The 1-year survival for heart transplants is approximately 87%, and the 5-year survival is approximately 77%.<sup>144</sup>

### Design Goals for Cardiac Tissue

The heart is a muscular organ that functions as a continuous pump through rhythmic contraction and relaxation cycles. The three layers of the heart are the epicardium, myocardium, and the endocardium.<sup>35</sup> The epicardium is the outer layer of the heart that provides a smooth layer to prevent friction while the heart is beating. The endocardium is the cell layer lining of the interior of the myocardial wall to create an antithrombotic surface and functions to interact with the circulating blood. The primary layer that forms the heart is the myocardium. The myocardium contains specialized cardiac muscle cells that are striated, involuntary

muscle with many branching cells that are joined by unique intercalated disks.<sup>35</sup> The intercalated disks are gap junctions that permit electrical communication that can pass impulses to a large area of the heart wall, thereby stimulating contraction.

The primary objective of cardiac tissue engineering in the near future will likely be the engineering of patches of myocardium to replace specific areas of diseased heart tissue. The challenge of engineering an entire heart with the ability to contract appropriately with separate chambers, adapt appropriately to physiologic stimuli, and respond to further cardiac disease progression appears too difficult to reach within a reasonable time given current technologies.<sup>145</sup> Another application of cardiac tissue engineering would be to use engineered cardiac tissue for reconstruction of pediatric congenital defects. The use of engineered tissue could potentially grow with physiologic development and prevent multiple surgeries for malformations such as single ventricles, atrial septal defects, or ventricular septal defects.<sup>145</sup>

The alternative cell-based therapies to cardiac tissue engineering are cardiomyocyte transplantation and the injection of marrow-derived cells, or other types of precursor cells, directly into myocardium. Cardiomyocyte transplantation has been studied in animal models by directly injecting fetal or neonatal cardiomyocytes into the injured myocardium. The positive findings of cardiomyocyte transplantation have included beneficial effects on the remodeling process in animal studies.<sup>146,147</sup> Despite moderate improvements in contractile performance related to the remodeling process, cardiomyocyte transplantation cannot replace scar tissue and restore synchronously contractile muscle.<sup>146,148</sup> The major limitation in translating this technology to human clinical trials would be the availability of cardiomyocytes. The intracoronary injection of marrow mononuclear cells, or other marrow-derived cells, in the setting of an acute myocardial infarction in humans, has thus far yielded no improvement in left ventricular function or minimal improvement in recovery.<sup>149,150</sup> Further studies, however, are ongoing.

### Cell Source

Marrow stem cells have been shown in some studies to differentiate into cardiac myocytes.<sup>151,152</sup> Primary cardiac cells are probably not a viable cell source in humans, because they are difficult to harvest and do not readily expand. Cardiac cells derived from rat marrow expressed proteins consistent with cardiomyocytes in vitro, and in a rat infarct model the transplanted cells improved the cardiac structure and function of the heart.<sup>153</sup> Rodent myocardial infarct animal models with cardiac cells derived from marrow have shown improved cardiac function and better ejection fraction with less remodeling of the left ventricle.<sup>152,153</sup> The challenge remains to develop an efficient method to isolate and to differentiate marrow stem cells into cardiac cells, particularly for human patients.

### Scaffold

The synthetic polymers and natural materials have been used for cardiac tissue engineering scaffolds. A study comparing PGA, gelatin, and polylactide copolymer with PCL using rat aortic

smooth muscle as the cell source found the PCL material to have better ingrowth of cells *in vitro* and improved cellular architecture as a cardiac patch compared to the PGA and gelatin.<sup>154</sup> The gelatin scaffold was noted to induce an inflammatory reaction, which was attributed to the xenogeneic (bovine) protein in the gelatin. Natural material using alginate-based scaffolds can form a three-dimensional structure with a uniform cell distribution of cardiomyocytes.<sup>155</sup> A graft composed of fetal cardiac cells in an alginate scaffold was implanted in a myocardial infarct model in rats and was found to undergo intensive neovascularization and had prolonged survival of the cells in the graft.<sup>156</sup> The alginate scaffold was almost completely degraded after 9 weeks, and it was well-invested with differentiated cardiomyocytes.<sup>156</sup>

Collagen gels have been used to form three-dimensional scaffolds that support cardiomyocyte cells. It has been shown that artificial myocardial tissue can be formed from neonatal rat cardiomyocytes inside a three-dimensional collagen matrix.<sup>157</sup> The constructs were prepared by adding a cell solution to preformed, commercially available collagen that was allowed to gel over 4 hours. The collagen matrix was found to support the attachment, proliferation, and synchronous contractions of the neonatal cardiomyocytes in the three-dimensional scaffold. The spontaneous and synchronous contractions were maintained for up to 13 weeks. Zimmermann<sup>158</sup> formed engineered heart tissue by mixing a cell solution of neonatal rat cardiac cells with collagen gel and Matrigel (Becton Dickinson, Franklin Lakes, NJ). After a few days, the constructs were placed in a custom-made stretch device to provide mechanical stimulation. The *in-vitro* tissue demonstrated histologic features of native differentiated myocardium and contractile characteristics of native myocardium. By exposing the cell constructs to stretch stimulation, the force of contraction was greater than twofold in the stretch group compared to the statically grown tissue.<sup>159</sup>

A novel method to engineer cardiac tissue has been developed by Shimizu. The concept is to use a temperature-responsive polymer called poly(*N*-iso-propylacrylamide) (PIPA Am) that covalently attaches to tissue culture polystyrene and supports the growth of cells. When the temperature is lowered to 32°C, the PIPA Am with the cells spontaneously detaches from the underlying polystyrene, with the cell-to-cell connections of the cultured tissues on the upper surface intact.<sup>160</sup> This material has been applied to rat neonatal cardiac cells to create sheets that can be layered together to form a three-dimensional myocardial tissue structure. Electrical and morphologic connections between the cell sheets have been demonstrated.<sup>160</sup>

## Bioreactors

The objective of bioreactor development in cardiac tissue engineering is to stimulate the growth of a three-dimensional structure that is conditioned by mechanical or electrical stimulation. A bioreactor system with a spinner flask causes turbulent flow that results in improved mixing for nutrient and oxygen delivery in comparison to static cultures.<sup>161</sup> Other designs of bioreactor designs have applied stretching or compression to cultured

cells. The exposure of myocardial cells to cyclic stretch resulted in improved proliferation and more uniform distribution of cardiomyocytes on the scaffold, as well as improved organization and more extracellular matrix.<sup>162</sup> The use of electrical stimulation with neonatal rat myocytes has been shown to induce structural differentiation similar to native myocardium, and an increased contractile response in comparison to nonstimulated cultures.<sup>163</sup> One important limitation to the growth of cardiac tissue for human applications is the thickness of the tissue. The thickness of human cardiac muscle is about 1 cm, whereas the thickness of cardiac tissues grown in bioreactor systems is only 100 microns.<sup>164</sup> Lacking a vascular network, cardiac tissue cannot grow significantly beyond 100 microns because delivery of nutrients and removal of waste is limiting.<sup>164</sup> Although bioreactor systems have resulted in great improvements, the next big challenge is to develop a construct and a bioreactor system that provide adequate perfusion to thicker cardiac constructs.

## Preclinical Studies

The preclinical data for myocardial tissue engineering remain in the early stages, with most investigators using neonatal or fetal rodent cells for evaluation of cardiac tissue engineering patches in animal models. In a study by Krupnick, a cardiac patch 4 mm × 4 mm × 2 mm was created with marrow MSCs as a cell source and a scaffold composed of a composite collagen and PLA material reinforced with PTFE.<sup>165</sup> The patches were implanted into the left ventricle of a rat nonfunctioning left ventricle model over a period of 4 weeks. The marrow cells integrated into the host and formed differentiated cardiac muscle by the cell morphology and cytoplasmic reactivity of anti-Troponin C. The authors suggested a cardiac patch could be implanted for human use to replace myocardial scar after infarction concurrently with coronary revascularization. However, the authors commented that a limitation of the animal model is that murine species are relatively resistant to arrhythmias.

Leor and colleagues engineered cardiac grafts with fetal cardiac cells using three-dimensional alginate scaffolds for the regeneration of infarcted myocardium.<sup>156</sup> The porous alginate scaffold was seeded with fetal cardiac cells and incubated for 4 days before implantation. The graft implantation was performed 7 days after myocardial infarct in a rat model and harvested 9 weeks after transplantation. The rats with the grafts demonstrated attenuation of left ventricular dilatation and no change in left ventricular contractility. In contrast, the control group developed significant left ventricular dilatation with progressive deterioration in left ventricular contraction. The cardiac grafts were found to have intensive neovascularization that contributed to the survival of the cells in the graft. The beneficial effects of the graft on cardiac contractility were not likely directly caused by contraction of the implant, because only a small fraction of the implanted cardiac cells survived after 9 weeks.

Zimmermann has reported the construction of an engineered heart tissue that can be constructed *in vivo* and can improve contractile function of an infarcted rat model.<sup>166</sup> The engineered

heart tissue was formed using a suspension of neonatal rat heart cells, liquid collagen, and Matrigel that was formed into circular molds and were subjected to mechanical strain. The myocardial infarct model in the rat was created by ligation of the left anterior descending artery. The engineered heart tissue and control, noncontractile grafts were implanted 14 days after infarct over the area of myocardial scar. The engineered heart tissue showed undelayed electrical coupling comparable to the native myocardium without evidence of arrhythmia at 28 days. The engineered heart tissue prevented further dilation and induced systolic wall thickening of infarcted myocardial segments, superior to control implants.<sup>166</sup>

### Future Challenges

The main challenges for cardiac tissue engineering are a reliable autologous cell source, and the vascularization of large tissues. Most of the studies utilize fetal or neonatal cardiac cells as a cell source. However, an autologous cell source from marrow or other cells is critical to advance cardiac tissue engineering for human applications. Clearly, it may be difficult to obtain viable cardiac cells from older patients with diabetes and other diseases because of impaired proliferative and functional capacity.<sup>167</sup> Methods to isolate, differentiate, and proliferate marrow-derived or other cells to produce cardiomyocytes need to be developed. After implantation, any cardiac graft will depend on neovascularization to maintain cell survival. Even if cell sheets can be used as layers to create a thicker construct, without a vascular network the cells would likely not survive. The potential applications for cardiac tissue engineering are a concurrent augmentation of scar tissue that occurs at the time of coronary revascularization and the reconstruction of congenital cardiac defects.

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## Corneal Tissue Engineering

A bioengineered cornea has the potential to restore vision when the loss is caused by opacification of the cornea. The cornea is essential to provide the proper anterior refractive surface and to protect the eye against infection and structural damage. The most common causes of opacification of the cornea are injuries or infection that lead to corneal scarring, abnormal shapes of the cornea, and congenital disorders of the cornea.<sup>168</sup> Diseases affecting the cornea such as measles, vitamin A deficiency, trachoma, and parasites are common causes of childhood blindness worldwide.<sup>168</sup> Corneal transplantation is the only widely accepted treatment for severe corneal diseases. The success rate of traditional allograft corneal transplantation is greater than 90% for patients with corneal disorders such as abnormal corneal shapes, traumatic corneal scars, and degeneration.<sup>169</sup>

### Objectives of Tissue-Engineered Cornea

The cornea is composed of an epithelial layer and an endothelial layer. The epithelium is a stratified squamous layer approximately four to six cell layers thick.<sup>168</sup> The tight junctions of the

epithelial cells provide a barrier to bacteria and prevent the entry of tears into the intracellular space. The basal layer of the epithelium contains epithelial stem cells that generate new corneal epithelial cells. The corneal endothelium is a hexagonal monolayer that maintains the fluid regulation of the corneal stroma. The endothelial layer maintains a high water content of around 78%, which ensures a cellular architecture that forms a transparent tissue.<sup>168</sup>

Although the primary treatment for severe corneal disease is corneal allograft transplantation, allograft rejection is the main cause of graft failure in corneal transplantation. The rate of rejection after 5 years has been reported at around 20%.<sup>170</sup> Topical corticosteroids are the mainstay treatment for immunologic rejection. Potential risk factors for immunologic rejection are increased stromal vascularization, prior blood transfusions, positive donor-recipient crossmatch due to preexisting lymphocytotoxic antibodies, young recipient age, larger grafts, and the presence of inflammation at the implantation site.

Synthetic corneal replacements, or keratoprotheses, provide an additional treatment option to those patients who are not candidates for allograft corneal transplantation. The design of the synthetic corneal prosthesis is a flexible material with an optically clear core that is surrounded by a microporous sponge rim.<sup>171</sup> The optical core functions to transmit light and provide refractive power, while the sponge rim is designed to permit fibroblast ingrowth to secure the device into place over the long term. Two FDA-approved keratoprotheses are available for clinical use.

The Dolman-Doane keratoprosthesis is double-plated, and is composed of a central core of polymethylmethacrylate (PMMA) that requires corneal donor tissue to form the rim.<sup>172</sup> The most favorable outcomes using the Dolman-Doane device are for patients with multiple failed grafts, repeated immunologic rejection of corneal allografts, and neovascularization of a corneal graft.<sup>172</sup> The postoperative course must be closely followed for retroprosthetic membrane formation, glaucoma, and retinal detachment.

AlphaCor, previously known as Chirila keratoprosthesis, is a one-piece prosthesis consisting of a central transparent optic region and an outer rim that is entirely manufactured from hydrophilic poly(2-hydroxyethyl methacrylate) or PHEMA. The porosity of the PHEMA sponge encourages biointegration with the host tissue to promote fibroblast ingrowth. AlphaCor has been successful in the treatment of corneal opacities that are not suitable for standard allograft corneal transplantation.<sup>173</sup> The complications of AlphaCor include corneal melting and the formation of retroprosthetic membranes.

### Cell Source

The cell source for corneal tissue engineering is corneal limbal epithelial cells or oral epithelial cells. Severe ocular surface diseases such as Stevens-Johnson syndrome, ocular cicatricial pemphigoid, and chemical or thermal burns often destroy the corneal epithelial stem cells.<sup>174</sup> In unilateral eye disorders, autologous



cultured corneal epithelial cells may be harvested from the unaffected eye and cultured as a sheet. Culture systems have been developed to proliferate and expand corneal epithelial cells that are harvested from primary tissue. In the earliest report of autologous cultivated corneal epithelial cells by Pellegrini, a biopsy from the limbus of the uninjured eye was obtained.<sup>175</sup> The autologous cultured corneal epithelium was implanted onto the cornea of two patients, and was found to restore the corneal surface with a significant improvement in visual acuity. A similar technique used by other investigators placed the biopsied corneal epithelial cells on denuded amniotic membrane in co-culture with a feeder layer of 3T3 fibroblast cells for 2 to 4 weeks.<sup>176</sup> After implantation of the tissue-engineered cornea, the patient had complete epithelialization with a significant improvement in visual acuity that remained stable.<sup>177</sup>

In patients who have bilateral ocular surface defects, the options are to use allogeneic cultured epithelial cells or epithelial cells from another tissue in the patient. The risk of using allogeneic cultured epithelial cells is an increased incidence of rejection and poor clinical outcome. In a small study by Koizumi, cultured allogeneic epithelial cell transplantation was performed in 13 eyes of 11 patients having total corneal stem cell deficiencies using amniotic membrane as a cell carrier.<sup>176</sup> This study resulted in all 13 eyes being free from epithelial defects after 48 hours, visual acuity being improved in all 13 eyes, and 10 of the 13 being restored to good function. Three of the eyes experienced epithelial rejection. To obviate allogeneic cells and immunorejection, epithelial cells from the oral mucosa have also been used for corneal applications.<sup>178</sup> A feasibility study was conducted in a rabbit model with an induced ocular surface injury. Oral mucosa biopsies were cultured for 3 weeks on a denuded amniotic membrane carrier. The cultured oral epithelial sheet had four to five layers of stratified cells with a similar micro-architecture to normal corneal epithelium.

### Scaffold

The scaffolds for corneal tissue engineering function more as carrier substances than as conventional scaffolds. The materials used as the carrier include amniotic membrane and fibrin-based materials. The characteristics that make amniotic membrane appealing for use as a carrier for the growth of epithelial cells are its ready availability, very limited immunogenicity, antimicrobial effect, and the fact that it resembles the basement membrane of the conjunctival epithelium.<sup>174</sup> The early reports of use of amniotic membrane for ocular surface reconstruction in rabbit animal models showed that resultant corneas were clear with minimal amounts of neovascularization.<sup>179</sup> The effect of the amniotic membrane suggested that it facilitated epithelialization without allowing host fibrovascular ingrowth, which can retard clarity of the membrane.<sup>179</sup> These preliminary animal data led to human studies, and amniotic membrane as a carrier substrate has proven to be successful in human corneal implants in multiple studies.<sup>177</sup>

Fibrin and fibrin-based carriers have also demonstrated success for corneal epithelial systems. Despite the advantageous

properties of amniotic membrane, the membrane remains permanently beneath the cultured epithelial cells. This may have some long-term effect on biocompatibility or pose a potential risk for infection. In contrast, fibrin and fibrin-based materials are biodegradable and will integrate with the surrounding tissue. Fibrin-fibronectin gel has supported human corneal epithelial stem cell growth and differentiation, with resultant expression of keratin cell markers consistent with corneal epithelium.<sup>180</sup> Human studies have demonstrated success in transplanting cultured corneal limbal cells on a fibrin carrier from the healthy eye to the contralateral eye.<sup>181</sup>

### Clinical Applications

Corneal tissue engineering has advanced to clinical studies. In patients with unilateral disease, a corneal biopsy from the healthy eye can be used as a cell source to obtain cultured corneal epithelial cells. In the setting of bilateral ocular surface diseases, a replacement cell source comprising autologous oral mucosal epithelial cells demonstrated feasibility in animal models, and this method has been applied to human corneal transplantation.<sup>177</sup> Two separate groups using autologous oral mucosal epithelium with different culture methods have performed corneal reconstruction in small case reports. The major difference in culture methods between the two groups is that one group uses a carrier-free epithelial cell sheet that is produced using temperature-responsive culture surfaces, while the other group uses amniotic membrane as a carrier for the epithelial cell sheet.<sup>177,182</sup> In the first group, four patients were enrolled in the study with one eye each undergoing transplantation.<sup>182</sup> Three of the four patients had undergone allogeneic corneal transplantation that had failed after 1 year despite systemic and local immunosuppression. The oral mucosal cells were harvested and cultured for 2 weeks on temperature-responsive cell-culture surfaces with 3T3 feeder cells.<sup>182</sup> The epithelial cell sheets were removed from the cell-culture surface by reducing the temperature and were transplanted directly onto the denuded corneal surface. Complete re-epithelialization of the corneal surface occurred within 1 week in all four treated eyes.<sup>182</sup> Over the 14-month postoperative period, the corneal surface remained transparent and there was significant improvement in visual acuity. In the second study, there were a total of six eyes in four patients with Stevens-Johnson syndrome (three eyes) or chemical injuries (three eyes).<sup>177</sup> Autologous oral epithelial cells were cultured for 2 to 3 weeks in the presence of 3T3 fibroblasts on a denuded amniotic membrane carrier. The epithelial sheet was transplanted onto the corneal surface and secured in place with sutures. The corneal surface had complete epithelial coverage by 48 hours, and visual acuity significantly improved over the postoperative period.<sup>177</sup> The corneal surface remained stable over a mean follow-up time of 13.8 months.

In a larger study of ocular surface reconstruction with cultured oral mucosal epithelial cells, mid-term results on a total of 15 eyes over a mean period of 20 months were reported.<sup>183</sup> The ocular surface remained stable and transparent in 10 of the 15

eyes, and visual acuity significantly improved in 10 of the 15 eyes. There were five eyes with small and persistent epithelial defects. Three of the eyes healed spontaneously, and two required reoperation.<sup>183</sup> These studies demonstrate that corneal engineering is a promising treatment for patients with severe ocular disease who fail transplantation, or for patients who are poor candidates for conventional corneal transplantation. The success of autologous oral mucosal epithelial cells for corneal tissue engineering applications obviates the need for immunosuppression and for acquiring corneal stem cells that may not be available when disease is bilateral.

## Disclaimer

The authors have disclosed no conflicts of interest.

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# VI Delivery of Transfusion and Transplantation Services

# Recruitment and Screening of Donors and the Collection, Processing, and Testing of Blood

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The process of supplying sufficient blood components and derivatives for patient needs is complex, highly regulated, and dynamic in adapting to the local and regional needs. This chapter focuses on the donation and collection process, from recruitment of donors to the receipt of products in the hospital inventory. Physician involvement is essential to this process.<sup>1</sup> An overview of the entire process can be seen in Fig 62-1. In many countries, there are dual systems: one for supplying blood for patient transfusion in the hospital and one for collecting source plasma for derivatives. This chapter reflects such an arrangement, with separate sections for collection of transfusable products and plasma for derivatives.

An understanding of the organization of blood services that are responsible for managing this complex process is addressed. Recruitment of blood donors is described, followed by detailed analysis of the donation process from the perspective of the donor and of the component prepared. Finally, donor adverse events are discussed.

## Organization of Blood Services

Approximately 80 million units of blood are collected worldwide each year by diverse blood organizations that vary based on local health initiatives, government intervention, and available resources.

The British Red Cross is credited with establishing the first organized blood donor service in 1921, identifying a panel of potential donors who would provide fresh blood at the time it was needed.<sup>2</sup> In 1935, the International Society of Blood Transfusion (ISBT) was established as a scientific and educational society to bring together professionals involved in blood transfusion and transfusion medicine worldwide (<http://www.isbt-web.org>). By 1935, a number of blood centers had been

established in Russia, at least two of which were providing blood for patient care.<sup>3</sup> Cook County Hospital in Chicago became the next blood bank in the world to store blood for future use in 1937.<sup>2</sup> The first community blood center was established in 1941 in San Francisco, California. Between that time and when the National Blood Service was established in the United Kingdom (1946) and the American Red Cross began its National Blood Program for civilians (1948), significant advances in transfusion medicine occurred. World War II (WWII) provided a great stimulus for the development of donor services and the banking of blood.<sup>4</sup>

## Organization of Blood Services in the United States

Blood collection for transfusion in the United States is accomplished by a heterogeneous system that has evolved since WWII. Close to half of the blood for transfusion is collected by the American Red Cross (see <http://www.givelife.org>). The other half is collected by nonprofit community blood centers, hospital blood banks, and collection centers operated by the armed services. Most of these nonprofit collection agencies are affiliated with a trade organization known as America's Blood Centers. Most blood centers and major hospitals are affiliated with the professional society AABB (see <http://www.aabb.org>).

Many hospitals have found it impractical to maintain hospital-based collections. Although hospital-based collection still exists, the increasing cost, external regulation, shortage of medical technologists, and variable supply and demand have progressively shifted collection activities to blood centers. In 2006, 95.1% of blood in the United States was collected by blood centers and only 4.9% by hospitals.<sup>5</sup> Even hospitals that collect blood usually do not perform donor testing and out of necessity maintain a supplemental blood contract with the local blood supplier. The US Department of Defense continues to operate an independent blood services organization for its hospitals as well as its military mission.

The concept of regionalization of blood services emerged from the assumption that a blood center should serve the surrounding areas that referred patients to major city medical



## Donor recruitment

*[collecting the right kind of blood at the right time to anticipate needs and avoid shortages and wastage]*

- recruitment organizations
- voluntary vs paid donors
  - donors of blood products for transfusion are usually voluntary
  - donors of plasma for fractionation are often paid (eg, Austria, China, Germany, USA)
- physician referral for autologous or directed donation

|

## Predonation information

*[intended to protect the recipient and the donor]*

- self exclusion
- donor interview
  - health history (cardiovascular, neoplasia, autoimmune disease, etc)
  - medication use
  - lifestyle risk factors (paid/received money for sex, incarceration, tattoos, etc)
  - travel history (malaria endemic areas, Chagas risk, UK BSE risk, etc)
  - TRALI risk assessment (previous pregnancy, received transfusion, etc)

directed physical examination (blood pressure, pulse, vein exam, temperature, etc)

hemoglobin or hematocrit screening (protein screen for source plasma donors)

platelet count (if required)

|

## Component collection

*[product selection depends on local needs, what the donor is eligible to donate, and maximizing the donation potential]*

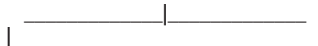
whole blood (450 to 500 mL)

apheresis collection

- RBC (up to 2 units)
- platelet (up to 3 units)
- plasma (up to 4 units)
- combinations of the above

additional blood specimens collected for donor testing

treatment of any donor reaction



## Component preparation/ testing

component separation

- RBCs
- whole-blood-derived platelets
- plasma
- cryoprecipitation

## Blood donor testing

ABO grouping/RhD typing

Screening for red cell antibodies

infectious disease testing

- serologic (HBV, HCV, HIV, syphilis, Chagas, etc)
- nucleic acid (HCV, HIV, WNV, etc)

**Figure 62-1.** The blood donation process: from donor to recipient.

- component modification
  - leukocyte reduction
  - gamma irradiation
  - pathogen inactivation
- quality assurance testing (platelet count, product volume, coagulation factor activity, etc)
- bacterial testing (platelets)
- plasma fractionation (IVIG, immune globulin, coagulation factor concentrates, etc)
- other testing (cholesterol, anti-HLA, etc)

### Postdonation information

- donor illness/self-deferral via confidential unit exclusion
- review/follow-up of any donor reaction
- positive infectious disease testing on subsequent donations ("look-back")
- information learned on subsequent donation that would have resulted in donor deferral (travel, history of disease, etc)

### Labelling of products for distribution

*[all legal and industry standards are met and testing is satisfactory]*

- inventory management to ensure sufficient stock when needed and to minimize outdate of products

### Blood products in hospital inventory

- selection of suitable product and appropriate use (crossmatch, clinical indication, etc)
- local component modification before transfusion
  - gamma irradiation
  - leukocyte reduction
  - plasma reduction/washing
  - pooling (whole-blood-derived platelets, cryoprecipitate, etc)
  - preparation for special procedures (aliquoting units, neonatal exchange transfusion, etc)
- documentation of recipients clinical information (antibodies, modifications, etc)
- evaluation and treatment of transfusion reactions
- notification of recipients in case of product recall or "look-back"

**Figure 62-1.** Continued

centers. This allows the entire geographic area to support the needs of its patients, whether they are cared for in the community or a referral center. By managing the blood within a region in a systematic way, waste can be reduced through the use of technological innovations and careful inventory management. Resource sharing arrangements help blood service organizations move blood around to different centers that are in need.

Since 2001, the annual blood transfusion rate in the United States has remained stable at about 50 units of Whole Blood (WB) or Red Blood Cells (RBCs) per thousand people. This was a change from the ever-increasing transfusion rate curve of the previous decade.<sup>5</sup> The amount collected in 2006 was 52.9

units per thousand total population.<sup>5</sup> This translates to 16.2 million units of WB/RBC collected that was transfused to 5.0 million recipients (an average of 3.0 units each).<sup>5</sup> The number of platelet concentrate-equivalent doses transfused has also remained stable at around 10 million.<sup>5</sup> In total, over 30 million total blood components were transfused in 2006. Essentially all components collected for transfusion in the United States are from volunteer donors; in contrast, the majority of plasma for fractionation is collected from remunerated donors, as discussed later. (The FDA definition of volunteer donor allows for remuneration as long as it cannot be directly converted to cash.)

## Organization of Blood Services Outside the United States

Almost all developed countries have adequate blood services, maintaining a full range of donor screening and quality assurance procedures, while producing most of the components needed in the region. However, there exists a wide variety of dissimilar organizational structures that are successful in meeting the transfusion needs of areas served. These models can be centralized or decentralized, governmental, military, private, hospital-based, or mixed. For the most part, the local transfusion system reflects the administrative system of each country. The World Health Organization (WHO) encourages strong governmental leadership in establishing national transfusion networks.<sup>6</sup> Overall, the provision of blood services worldwide is heterogeneous and varies both with historical development, the socioeconomic development of the region, and the influence of national scientific and political factors.<sup>6</sup>

Recruitment is sometimes carried out by independent organizations that have a special relationship with the collecting agency, such as a country's national Red Cross. The International Federation of Blood Donor Organizations was established in 1955 as a support network for donor recruitment (see <http://www.fiods.org>). More than 155 nations participate. The goals of the organization include member state self-sufficiency in blood from voluntary, unpaid blood donors; improvement in safety; and, in turn, confidence in the national blood supplies through development of minimum standards for donations, inspection, and quality assurance.

In 1975, the 28th World Health Assembly passed a resolution recognizing the value of voluntary blood donation and called on member states to promote national blood transfusion services based on voluntary, unpaid donations.<sup>6</sup> Voluntary donation has been a goal for some time, as it is perceived to be a safer alternative. In 2002 the European Union approved legislation that established comprehensive standards for blood products that included a requirement for voluntary donation.<sup>6</sup> The WHO Global Database on Blood Safety 2004 report collected data from 172 countries representing 95% of the world population.<sup>7</sup> The report shows that there is still inadequate collection in developing countries, which account for 80% of the world's population, but collect only 45% of the world's blood supply. This "gap" is often closed by the use of paid donors. Since 2001, there have been tangible improvements in the proportion of volunteer donors, but family/replacement donors and paid donors are still a significant source of blood for transfusion in many developing and transitional countries. More than 2.2 million units were collected from paid blood donors in 2004. Nevertheless, countries are increasingly moving toward voluntary blood donation. In 2001-2002, 63 countries were collecting less than 25% of their blood from voluntary, unpaid donors; by 2004, this had decreased to 46 countries. Particularly encouraging was the increase from 25% to 47% in the proportion of total donations collected from voluntary, nonremunerated blood donors in developing and transitional countries. During the same period in developed countries, 92%

of donations were from voluntary unpaid donors, as compared to about 67% in developing and transitional countries. The ultimate goal of the WHO and its supporters is that countries should collect all blood from unpaid volunteers. By 2002, 39 countries had achieved 100% unpaid voluntary blood donation, of which five were developing countries. By 2004, this number had risen to 50. During this time, a total of 60 countries reported an increase in the percentage of blood donated by voluntary unpaid blood donors, while 41 countries maintained the same level. Unfortunately, 37 countries showed a decline in the percentage of blood donations from nonremunerated, voluntary blood donors.<sup>7</sup>

The WHO recommends that, at minimum, all blood for transfusion should be screened for human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), and syphilis. In 2004, 41 out of 148 countries (28%) that provided data on screening for these four transfusion-transmissible infections were not able to screen all donated blood for one or more of these infections.<sup>7</sup> Even in countries that are able to screen essentially all of their blood, the risk of transfusion-transmitted infection varies. For instance, in 2002, the residual risk of infection from *screened* blood was 10 to 20 times greater in Latin America than in certain industrialized countries.<sup>6</sup> However, some progress is being made globally in decreasing the amount of blood transfused without testing; the number of units for which the four WHO-recommended infectious disease tests were *not performed* decreased from 6 million in 2002 to 1.5 million in 2004. The most marked reduction was seen in the African region where the number of units with tests *not performed* was reduced from more than 1 million in 2001 to 380,000 in 2004.<sup>7</sup>

Developing countries often struggle to provide the same level of safety as countries with established blood procurement systems. The costs of procurement and testing of the blood are often prohibitive. For instance, in sub-Saharan Africa, despite a long history of blood transfusion,<sup>8</sup> blood services are fragmented. Blood is sometimes in short supply and safety is variable. Fewer than 3 million collections were performed in 2004 for a population of 700 million. Of 40 countries in sub-Saharan Africa, 28 countries have yet to implement national quality systems in their blood transfusion services.<sup>7</sup> The economic disparity among the countries of the world in blood procurement and testing may be greater than any other area of health care. Some industrialized countries have implemented a "precautionary principle" that has meant high costs for marginal safety improvements when measured by standards such as quality adjusted life-years.<sup>9</sup> In stark contrast to the "precautionary principle," a portion of the world's blood supply is still transfused without testing. In addition, when testing can be performed, there may not be resources to notify and counsel donors about positive test results, such as HIV. This provides an opportunity for the world community to make a great impact on global health through collaboration and collegiality. In response, the WHO established the Global Collaboration for Blood Safety in 1994, a forum which is a voluntary partnership of internationally recognized organizations, institutions, associations, agencies, and experts from developing and

developed countries that are concerned with the safety of blood and blood products (see <http://www.who.int/bloodsafety/gcbs>).

## Recruitment of Blood Donors

The first task of a regional blood center is to recruit adequate numbers of donors to provide for patient needs. Traditionally, this requires a strong association between the blood center and the population in an area, as well as the cultivation of an altruistic spirit within the community.<sup>10</sup> As described by Titmuss, the act of giving blood clearly demonstrates the integration of individuals into society.<sup>11</sup> However, as discussed throughout this chapter in more detail, a fragile balance exists between blood supply and blood demand in the United States. A recent report calculates that only 111 million individuals in the US population are eligible to give blood based on age and exclusionary factors for established risks. Previous calculations estimated this number at 177 million eligible blood donors.<sup>12</sup> These findings come at the same time the baby boomers, a mainstay of the donor base, approach those years when exclusionary factors increase and only a small fraction of eligible donors donate the “gift of life.” The recruitment of blood donors is now the most challenging task at the blood center.

Many studies have attempted to explain why people do, or do not, become blood donors. Other studies have sought to determine the motivation to repeat the donation process. These studies have evaluated demographic and sociological/psychological characteristics as well as donor motivation for giving or not giving blood. The challenge is to apply these findings to the everyday recruitment of blood donors.

### Donor Demographics

The demographics of current blood donors provide the blood center with insights into where and to whom they might focus marketing and recruiting efforts. As expected, individuals who are integrated into American society at upper socioeconomic ranges are more likely to volunteer in community efforts, give money to charitable causes, and donate blood than individuals in marginalized populations who are struggling at lower socioeconomic levels.<sup>13–15</sup> Donors who are older ( $\geq 50$ ) and who have more education (college graduates) are more likely to return as repeat donors than those who are younger and who have not gone to college.<sup>16–20</sup> Thus, blood donor organizations are challenged to recruit a more diverse group of donors who are not represented in the traditional donor pool.

### Donor Motivation

The earliest studies showed that the concept of altruism has been associated most often as the reason for giving blood.<sup>21,22</sup> In volunteer blood programs across the globe, the unselfish act of giving blood for the welfare of others remains the centerpiece of blood donor recruitment initiatives. But other motivators include the concepts of community need, and social

pressure to conform to expectations or desires of an individual or group. People donate because someone asked them to, they have heard about an emergent need for blood, or because others are doing it. Convenience to donate has also been identified as an important factor and blood centers understand the value of strategically located fixed donation sites and the need for mobile operations.<sup>23</sup>

More recent studies have examined the role incentives have on donor motivation. Blood credits (eg, credit toward blood units required by members of a family or community group) were most attractive to donors as incentives, as well as cholesterol screening and a prostate-specific antigen (PSA) screening in men. In general, small incentives or tokens of appreciation were more likely to appeal to younger donors than older donors.<sup>24</sup> In a follow-up study, first-time donors were positively influenced by incentives, but this finding was also related to the younger age of these donors.<sup>25</sup> In another study, first-time donors were more likely than repeat donors to be encouraged to donate and less likely to be discouraged if offered cash, event or lottery tickets, or merchandise. Donors attracted by cash were more likely to have a risk for transfusion-transmitted infections. Medical testing and blood credits were attractive to both first-time and repeat donors.<sup>26</sup>

Younger and first-time donors also decided to donate because of a family member, friend, or coworker. Younger and first-time donors were also motivated to donate because of testing for infections. Donors with a college degree were less likely to donate to improve their health (eg, by receiving PSA or cholesterol screening) or because of family or peer influence.

More than 90% of respondents to a questionnaire administered to donors of varied ethnic backgrounds cited a desire, responsibility, or perceived duty to help others as an important or very important motivator to donation. Being asked to participate in a work-related blood drive was also an important motivator and not being asked was a deterrent. Getting the results of a health screen appealed to many donors. More than 50% of respondents did not find any of the incentives (gifts, tickets, time off work, reward) important at all in their decision to donate.<sup>27</sup>

In a recent Canadian study, the importance of altruism as a motivator to donation was noted along with family and social influences.<sup>28</sup> Blood centers need to identify potential donors who are more likely to be motivated by the message to “make it available for themselves if required” as well as potential donors more likely to respond to an appeal to “make it available for someone close to you.”<sup>29</sup> In another randomized, controlled trial donors received information about the indication for transfusion of their blood and the recipient’s status. Donors were enthusiastic about receiving this information. However, no significant increase in donation frequency could be demonstrated.<sup>30</sup>

### Deterrents to Donation

There are many deterrents to blood donation. Some deterrents, such as inconvenience, perceived incompetence of personnel

performing blood collection activities, and the lack of cleanliness of a given facility or operation are clearly within the control of the blood center. But, some deterrents, including the perception that blood donation is not important, the belief that one can contract a disease by donating, and the desire to remain ignorant of any positive results from infectious disease testing on the donation are more difficult to overcome.

The most frequently mentioned negative motivator to donation is fear,<sup>28,31,32</sup> including fear of needles, seeing blood, weakness, dizziness, and discomfort. In some individuals, these types of fears may never be overcome. Education programs tailored to overcome fear and heighten the awareness of need may be helpful.

Medical disqualification is also a frequently given reason for nondonation. However, several studies have shown that many perceived reasons for nondonation have been invalid or imagined.<sup>21,31</sup> At least for some individuals, invoking a medical disqualification is more appealing than admitting to some type of "fear" relative to blood donation.

Treatment by blood center staff and the donor's perception of staff competence, coupled with donor sense of well-being during and after donation influence the likelihood of donor return, although highly committed donors are generally not deterred by the occasional bad experience during donation.<sup>15,16,23,33</sup> Donation-related symptoms including dizziness, nausea, and fainting are a significant reason for donor nonreturn.<sup>34</sup> Several strategies have been proposed to mitigate symptoms and increase donor returns.<sup>35,36</sup>

Temporary deferral has a significant impact; deferred donors return less frequently than nondeferred donors and are more likely to lapse from donation.<sup>37-39</sup> In a further study, repeat donors who reported a previous temporary deferral were more likely to lapse, but the association disappeared when other factors such as accessibility, satisfaction with last donation, and perceived need for blood were adjusted.<sup>33</sup> Of interest, several lapsed donors incorrectly viewed themselves as permanently deferred for temporary conditions such as low hemoglobin or hematocrit.<sup>32</sup>

### Sociological and Psychological Theories of Blood Donation

Many theories exist about donor motivation, and several excellent reviews are available with more detail on this complex aspect of blood donor recruitment.<sup>37,40-42</sup>

Opponent-process theory has been used to explain why some individuals repeat the process of donation and become committed, habitual blood donors. Donors experience a "warm-glow" after donation, which may represent an opponent process in response to negative feelings experienced before and during initial donations.

Attribution theory suggests that people who have taken an action (eg, donating blood) without external coercion or large reward, are likely to attribute to themselves a predisposition toward that action. Once they have attributed such a tendency to

themselves, or once they decide that they are the kind of people who do such things, they are more likely to act in ways consistent with that attribution in the future.

In the model of commitment, four processes are proposed: coping with and neutralizing the negative aspects of donation; developing internalized motives for donation and integrating them into one's self-concept; developing a behavioral intention to continue giving blood; and finally, developing a self-sustaining habit of donation.

The theory of reasoned action states that all behavior is preceded by a behavioral intention that can be measured by seeking an estimate of the probability of acting on that specific behavior. Intention is a function of two additional factors, the individual's attitude toward performing the act and the perceived expectations of others for what one should do in a particular situation. This theory has been, in general, effective in explaining donor action related to blood donation. For example, donors who verbally expressed their intention to donate when recruited were more likely to attend the drive than those who were merely reminded that a drive was occurring. Likewise, when donors are informed of a blood mobile and also sent a checklist of dates to which they could commit (forming an intention), they were more likely to give blood when compared to donors who were merely informed of the drive through the media.

The attitudes of donors and nondonors in three areas including affect, cognition, and behavior have been reported.<sup>43</sup> In terms of affect, donors were more likely to indicate that blood donation made them feel generous, assured, relaxed, and useful. Nondonors were more likely to indicate that donation made them feel uncomfortable and ill. On cognition scores, more nondonors believed that blood donation was dangerous and appeared to know little about the process. Donors were more likely to cite that blood donation is worth any inconvenience and is an important civic duty. For behaviors, donors ranked more favorably those behaviors that reflected the donation process. The authors concluded that blood donor behaviors may be more strongly determined by affective and emotional processes rather than by carefully reasoned decisions.<sup>43</sup>

It appears from the recent literature that the model most likely to predict future blood donation behavior is the theory of planned behavior.<sup>44, 45</sup> This model postulates that behavior can be determined by intentionality, which in turn is affected by attitude (positive or negative evaluation of the behavior), subjective norm (perception of social pressure), and perceived behavioral control (perceived ease or difficulty in performing the behavior). Several studies involving nondonors concluded that this model predicted 31% to 72% of the variance in intention to future blood donation. In a recent study<sup>46</sup> based on the theory of planned behavior and experienced donors, 65% of the variance in donation intention and 50% of the variance in attitude was accounted for. Self-efficacy showed the strongest positive relationship to donation intention, followed by attitude, subjective norm, satisfaction, and personal moral norm. Prior vasovagal reactions related negatively to intention to donation.<sup>46</sup>

## The Collection Process for Blood Components for Transfusion

### Donor Evaluation

The twofold purpose of blood donor screening is to minimize the risks to both the blood recipient and the donor. Donors should be informed as early as possible about all aspects of the donation procedure and about the importance of critical self-evaluation and self-exclusion for those who do not qualify. Prerecruitment information about donor qualifications and predonation information about risk factors for transmitting infections through transfusion should lead to self-deferral by some donors. When the donor presents for donation, written educational material is given, including a list of deferral medications. Acceptable donor age is 17 and above in most centers, but there is considerable variation in practice. Recent efforts have convinced governments to allow 16 year olds to donate. Experience with healthy donors over the age of 65 has generally been favorable; thus, the need for an upper age limit is questionable.<sup>19</sup>

Answers to donor history questions in a confidential setting provide another opportunity to obtain information about potential risk. Only 3 years after the founding of AABB, a list of 21 diseases and conditions were introduced on the “donor record card” that was intended to be used nationwide for screening criteria.<sup>47</sup> This was based more on the medical judgment of the day than on clear-cut data or evidence. Many of the current medical deferrals are still based less on data and more on a combination of opinion, tradition, and “conventional wisdom.”<sup>47</sup> With the advent of HIV, a second phase in the development of the questionnaire was based on epidemiologic evidence and included direct questions about sexual risks. The FDA stressed a face-to-face interview, rather than filling out a questionnaire, in hopes of ensuring complete honesty in answering the questions. Such data as history of hepatitis and possible exposure to HIV are used to deter donations from persons with potentially infectious exposures. This is done in order to reduce the pretest probability of finding true positive results during laboratory testing. In other words, the precollection donor screening process reduces the amount of blood collected that can transmit infectious diseases. Although laboratory testing is very sophisticated, it can never completely eliminate the risk of an infected unit. Reducing the number of infectious units that make it to the testing laboratory will reduce the number of infectious units that make it through the testing process undetected (ie, false-negative test results).

The donor questionnaire is a dynamic document that is updated as new risks become apparent.<sup>47,48</sup> The interview process is ineffective if it becomes too onerous or lengthy. If there are too many questions, important issues about high-risk behavior might be obscured, and the ultimate purpose of the interview process would be defeated. In the United States, a Uniform Donor History Questionnaire (UDHQ) has been created to standardize criteria used for all tissue and blood donations and reduce the complexity, burden, and possible confusion. Donors

are queried using broad-based capture questions to make screening more efficient (see <http://www.aabb.org>). Certain questions are designed to eliminate the need for further exploration of the subject if the answer is no. The questions are asked in reverse chronologic order. Questions start with the day of presentation, proceeding backwards and query about donor health status, travel, medications, and other items associated with increased risk, either to donor or recipient (eg, “in the past 48 hours . . .” “in the past 6 weeks . . .” “in the past 12 months . . .” “have you ever . . .”). Some medications are cause for deferral because the indication for use increases the donor’s risk in donating (such as antiseizure medications) and others are cause for deferral in order to protect blood recipients (for example, the use of potentially teratogenic medications such as isotretinoin). Still other medications might suggest a condition where the donor may not be able to give consent (eg, Alzheimer’s disease).

A comprehensive treatment of the screening process has recently been published.<sup>48</sup> Screening questions address items such as the following:

- Current state of health.
- Medication use (including aspirin).
- Pregnancy.
- Recent blood donations or immunizations.
- Receipt of a transfusion/transplant/graft.
- Sexual practices that may increase the risk of HIV.
- Body piercing or tattoos.
- Incarceration.
- Exposure to hepatitis.
- Travel to areas where certain diseases are endemic (eg, malaria, Chagas’ disease).
- Use of clotting factor concentrates.
- Injection of any drugs not prescribed by a physician.
- History of diseases such as malaria, Chagas’ disease, babesiosis, cancer, cardiovascular disease, a bleeding condition, or a family history of Creutzfeldt-Jakob disease.

During the interview, prospective donors might become aware of disqualifying factors in the health histories but feel too embarrassed or coerced to admit to disqualifications. Provisions must be made at all collection sites for a person to exit at any time without examination and the opportunity must be provided for the donor to easily indicate that collected blood not be used for transfusion. Mechanisms have been established for confidential unit exclusion that provide for this either at the donation site using a confidential form or barcode, or by anonymous telephone call after donation.

Donors also receive an abbreviated physical examination including monitoring for symptoms of current disease processes, body temperature, heart rate and rhythm, and the ability to comprehend the screening questions and give consent. Consent for donation should also include the possibility that the donor will be listed in a deferral registry if the history or testing preclude donation. In some areas, laws may require that public health authorities be notified when donors test positive for certain diseases.

A blood sample is tested before donation to make sure the hemoglobin level is at least 12.5 g/dL or a spun hematocrit is at least 38%. This screening for anemia is the minimum requirement for allogeneic whole blood donation in many countries. Other automated procedures such as red cell, platelet, and plasma collection by apheresis technology may require more rigorous hemoglobin cutoffs and have minimum height and weight guidelines. Blood centers in the past most often measured red cell mass using copper sulfate, but recently more objective measurement methods that measure hemoglobin or hematocrit have been employed. In the copper sulfate method, a drop of capillary blood from a fingerstick sample is dropped into a copper sulfate solution with a specific gravity of 1.053. Sinking of the drop indicates a hemoglobin of 12.5 g/dL or above. The copper sulfate method, although well accepted for decades, suffers from problems with both specificity and sensitivity. Because of the former, donors who fail are often retested with a spun hematocrit. This test can also err in accepting individuals who are proven anemic by a venous sample taken at the same time.<sup>49,50</sup>

A prospective donor with a diagnosis of chronic, degenerative, or infectious disease should be deferred from blood donation. Donors whose histories carry significant risk and those who have tested positive for infectious diseases are placed on deferral registries. For some deferrals, “re-entry” pathways back into the donor pool have been made available when the risk is no longer present. On occasion, donors attempt to donate in spite of permanent deferral. Reasons include a desire to obtain results of infectious disease testing, to receive credit for community service, a misunderstanding about the reason for deferral, and erroneous recruitment by the blood center staff.<sup>51</sup>

The effectiveness of the donor interview in deferring individuals who have risk factors that could affect the safety of the product has been a subject of investigation. Most recently the Retrovirus Epidemiology Donor Study has shown areas where improvement is needed. Younger donors (less than 25 years of age) have more behavioral risk factor than older donors. Educational reinforcement is needed for this group.<sup>52</sup> Most donors skim the educational material and fail to assimilate the information. Thus, more effective materials are needed. However, some high-risk donors seem resistant to any attempt at education and test-seeking behavior is a particular problem.<sup>53,54</sup> Computer-assisted self-interviewing (typically using a touch screen) has become an increasingly popular vehicle for donor screening. Data from this group suggests that it is probably able to reduce the number of high-risk donors by increasing self-deferral.<sup>55</sup>

Once the donor has been appropriately evaluated to ensure that the risk to the donor and to the recipient is acceptable, then blood collection may occur.

## Blood Collection

This section first addresses the traditional collection of whole blood and its separation into components, followed by discussion of newer automated apheresis technology.

## Whole Blood Collection

Despite the many technologies for automated collection, some blood centers—indeed entire countries—are able to produce the bulk of needed inventory from whole blood collections. A typical whole blood collection takes 45 to 60 minutes, including the interview, physical examination, aseptic scrub, antecubital venipuncture, and monitoring for postdonation reactions in a canteen area where refreshments are offered. The blood draw itself should be accomplished in approximately 10 minutes, with periodic agitation to mix the fresh blood with the anticoagulant in the draw bag to reduce clotting. The tubing is clamped before the needle is removed so that air is not drawn into the blood bag. Blood samples are taken into pilot tubes for testing. This is normally done from the blood tubing at the conclusion of the donation or from a diversion pouch filled from the first blood collected, a process that captures contaminating skin organisms. The tubing is sealed and the needle is disposed of without recapping. The donor holds firm pressure over the venipuncture site and then a pressure bandage is applied. The donor is instructed to keep the pressure bandage on and avoid strenuous activity for a prescribed amount of time, to increase fluid intake for the next day or two, and to spend at least 15 minutes in the canteen area. Reactions usually occur at the end of donation in the donor chair or in the area where refreshments are served. If the donor leaves immediately against advice, risk of injury might be increased (see “blood donor adverse events” below). Staff monitoring the postdonation canteen area must be vigilant in assessing for signs of reactions and proactive in preventing fall injuries.

Whole blood collection has the longest cumulative experience and requires fewer resources than automated collection. For countries in the process of developing simple, reliable collection networks, whole blood is the logical first step, with or without component separation.

## Component Separation

Whole blood may generally be stored for up to 21 days. However, the vast majority of whole blood collected in countries with well-established blood systems is separated into components. This allows longer shelf life, better inventory management, and more choices and resources for patient care. Whole blood may be separated by centrifugation into RBC, platelet, and plasma components according to density. Using a “closed” system that is not ever open to outside air is important to prevent contamination with microorganisms during collection and component separation. Centrifuge-separated components are transferred from the original whole blood draw container to satellite bags through integrally connected plastic tubing. Adding nutrients and saline to the red cells permits storage at 1 to 6°C for up to 42 days. The preservative solution may be held sterilely in one of the integral transfer bags until the plasma is separated into a third bag after centrifugation. Then the solution may be added back to the red cells in the original bag.

Platelets may be separated from the whole blood or allowed to break down without being separated. In Europe and many areas of the world, “buffy-coat” platelets are produced by using

a “hard” spin initially to separate all cellular elements from the plasma. Then the buffy coat containing the platelets, white cells, and a significant amount of red cells is further processed to separate the platelets. In the United States, an initial “soft” spin separates platelet-rich plasma from the red cells and then a “hard” spin separates the platelets from the plasma. Additional information about platelet preparation and storage may be found in Chapter 12. Platelets are typically stored from 5 to 7 days at controlled room temperature before expiration. In 2006, the mean age of whole-blood-derived and apheresis platelets at the time of transfusion was 2 and 3 days respectively.<sup>5</sup>

Plasma separated and frozen within 8 hours is called Fresh Frozen Plasma (FFP), while plasma frozen within 24 hours after phlebotomy is known as FP24. Each contains coagulation factors in quantities adequate for most patient indications. FP24 represented only 15% of the 4 million units of plasma transfused in the USA in 2006.<sup>5</sup> However, with the recent concern about transfusion-related acute lung injury (TRALI) and the exclusion of women who have been pregnant and possibly transfusion recipients from plasma donation, the amount of FP24 transfused is expected to increase dramatically. This is because blood centers are attempting to make plasma from all eligible donors of the needed types, even though the plasma logistically cannot always be frozen within 8 hours.

After separation from the red cells, plasma may be pooled and treated with solvent and detergent to decrease the risk of transfusion-transmitted disease. In addition, the pooling enables quality control of coagulation factors per lot and the dilution dramatically decreases the risk of TRALI.<sup>56,57</sup> Some countries in Europe rely on this process for the vast majority of plasma transfused.<sup>56</sup> Solvent/detergent-treated plasma is approved for transfusion in the United States; however, there are currently no companies producing this product.<sup>57</sup>

Preparation of cryoprecipitate involves thawing frozen plasma at 4°C in a circulating waterbath, followed by centrifugation so that the supernatant plasma can be drained into an integrally attached storage bag. The remaining cold-precipitated material is then refrozen at  $\leq -18^{\circ}\text{C}$  and stored for up to 1 year. A total of 1.2 million units of cryoprecipitate were prepared in the United States in 2006, 98% of which were produced by blood centers.<sup>5</sup> In some regions in Europe, cryoprecipitate is not produced, because the use of factor concentrates for inherited bleeding disorders and the use of plasma or fibrinogen concentrate for hypofibrinogenemia have proven satisfactory.

### Leukocyte Reduction

Leukocyte reduction is accomplished by passing WB or RBC units through filters that remove white cells (WBCs). Ideally, this should be done as soon after collection as possible but no longer than 72 hours. Leukocyte reduction has been shown to be more effective when performed before storage because some of the adverse effects of transfusion are caused by factors produced or released by leukocytes during storage. Although leukocyte reduction can reduce untoward events, significant amounts of WBC still remain

in leukocyte-reduced products (up to  $5 \times 10^6$  WBC/unit in the United States and  $1 \times 10^6$  in Europe). In the United States from 2004 to 2006, there was a 9.9% decrease in transfused prestorage leukocyte-reduced units and a 52.7% decrease in transfusion of poststorage leukocyte-reduced units.<sup>5</sup> This decrease reflects the decrease in use of whole-blood-derived components. Automated apheresis collections often have technology that produces leukocyte-reduced products during the collection process.

### Automated Collection

Over the first decade of the 21st century, advances in technology combined with a desire to optimize donations has led to a dramatic increase in automated collection of blood components. The collection of platelets by apheresis has increased coincident with the greater availability of automation and implementation of bacterial testing of platelet products. In the United States in 2006, apheresis-derived platelets increased 9.0% from 2004 (8,338,000 to 9,092,000); simultaneously, the transfusion of whole-blood-derived platelets decreased by 15.7% (1.5 million units to 1.3 million).<sup>5</sup>

Choice of which automated product(s) to collect depends on a number of factors, including blood type, height, weight, gender, risk of having antibodies that could cause TRALI, platelet count, hematocrit, the available collection technology, and the time the donor has available to donate. Minimum donor qualification requirements for the various automated collection methods have developed historically—often independently. Therefore, some procedures have minimum requirements and length of deferrals that may differ from other procedures. A 2-unit RBC product is produced by an apheresis procedure where 2 RBC units are collected at the same time, while returning donor plasma and often additional saline solution. In the United States there is a 112-day deferral for donation of a 2-unit RBC product that also precludes platelet and plasma donation on some instrumentation. At the same time, a donor of a combined automated platelet, plasma, and red cell collection is deferred only 56 days for red cells, but may often continue donating platelets and plasma again during the red cell deferral.

Selection of which product(s) to draw from a given donor may be complex in a center with many choices. Obviously, the first concern is which product is most needed for patient care at that time. In addition, an underlying theme in blood banking is to maximize collection of universal donor group AB plasma and universal donor group O red cells. Many centers avoid collection of RBC units from group AB donors because many of these RBC units are wasted, because only group AB recipients may use this blood. Recently, risk of antibodies that may cause TRALI has also disqualified donors from plasma and possibly platelet donation who are at risk of having these antibodies. Finally, there may also be minimum height, weight, and hematocrit requirements that vary by technology and by gender. Therefore, a large male with a high platelet count might donate three units of platelets by apheresis, while a small woman who has TRALI risk might donate only an RBC product.

With proper attention to donor interviewing and care of the donor, the blood donation process can be a pleasant and safe



experience for the donor, while providing a safe and efficacious product to the recipient—the goal of the whole process.

## **Testing**

Blood specimens drawn from the donor at the time of collection are sent for blood typing and infectious disease testing. Many hospitals that still collect blood find it more economical to send out the testing and receive the results electronically. Bacterial testing of platelet units is sometimes also done centrally.

### **ABO/Rh Testing**

Donor centers routinely test blood from each donation for ABO and Rh(D) type (and K in some countries). “Forward” typing occurs by addition of antibody directed toward the A, B, and D antigens on donor red cells to obtain the blood type. “Reverse” grouping is performed by adding donor plasma to reagent red cells bearing A or B antigens to detect naturally occurring donor antibody to A and B antigens. Algorithms ensure concordance of forward and reverse typing.

“Rh-negative” donors lacking D antigen undergo an additional step in the blood screening process to determine if their cells possess the weak-D antigen. As a rule, when in doubt, blood centers err on the side of calling a donor D positive, to avoid the chance of giving Rh-positive blood to an Rh-negative patient. At the transfusion service (recipient) level, the patient is typed and, if in doubt, the transfusion service errs on the side of calling recipients Rh-negative, so they will not inadvertently be stimulated to make an alloantibody to D. The processes are optimized for these two divergent purposes. This is the reason that discrepancies may sometimes be discovered when blood donors become patients and their D typing may appear to change from positive in the blood donor setting to negative in the blood recipient setting.

### **Antibody Screening**

Donor plasma is also added to reagent red cells bearing an array of clinically significant red cell antigens. This is to screen for unexpected alloantibodies to epitopes in the Rh, Kell, Kidd, Duffy, and other antigen systems. Plasma from donors bearing alloantibodies is not used for transfusion; however, because RBC units contain so little plasma, these may be made available from donors with alloantibodies with the appropriate labeling. The screening of the donor for alloantibodies eliminates the need for the transfusion service to screen for donor antibodies to red cells and eliminates the need to perform a “minor” crossmatch (donor plasma mixed with patient red cells).

### **Infectious Disease Testing**

Serologic and nucleic acid amplification testing (NAT) for infectious disease is carried out in most developed countries. The exact tests performed in a center depend on the incidence of the diseases in the population and also on availability of resources for testing. Testing requirements have changed frequently in recent years. Currently, infectious agents for which serologic screening is performed in the United States include HIV-1/2, HCV,

HBV (HBsAg; HBe), human T-cell lymphotropic virus, types I/II (HTLV-I/II), Chagas’ disease, and syphilis. NAT is performed for HIV-1/2, HCV, HBV, and West Nile virus. Supplemental or confirmatory testing is often performed when licensed tests are available. This is useful for donor counseling and to potentially allow the donor to be eligible for a “re-entry” protocol (which in the United States must conform to FDA guidance). Generally speaking, a donor is deferred even if the screening test is known to give a false-positive result. This is because the test has become uninformative in that donor—ie, it cannot discriminate whether the donor has the disease. This often happens when blood testing laboratories change from one testing platform to another (eg, switching to a different instrument manufacturer). It is not unusual to have a higher number of deferrals concurrent with the implementation of the new technology because of the false-positive tests. Some countries—such as England—may be more donor-centered in their testing algorithms. For instance, if a donor is known to be false positive on a certain screening platform, subsequent specimens may be routed to an alternate platform, rather than deferring the donor. If a donor is known to have visited an area in which malaria is endemic, the donor might become eligible to donate after a short deferral period if a supplemental malaria test is performed, rather than be deferred as long as US donors. In 2006 in the United States, approximately 1% of allogeneic whole blood donations had positive results on infectious disease screening tests, resulting in the destruction of 151,000 units of blood.<sup>5</sup>

In addition to serologic and NAT assays, bacterial culture is becoming widely used to test platelet products. Because platelets are stored at room temperature, rather than refrigerated or frozen, there is a greater chance that bacteria in the product might grow. These organisms may come from normal skin flora that contaminate the bag during phlebotomy or they may be transiently circulating in the donor’s blood during collection.

## **Distribution**

When a blood component has successfully completed processing, testing, and record review, then it is “labeled,” meaning that it has met all standards and is ready for infusion. Many countries are adopting ISBT 128, an identification system developed by ISBT that incorporates comprehensive bar-code labels intended to set a global safety standard for the identification, labeling and information processing of human blood, tissue, and organ products across international borders and disparate health-care systems.<sup>58</sup>

The vast majority of a typical area’s blood supply is stored in hospitals for use when it is needed, rather than at the blood center. Availability and turnaround time are key components for determining allocations to each hospital; these depend on usage patterns and transportation distance from the blood center. Even a very small hospital that is a great distance from its blood supplier might have 10 or 20 RBC units on its shelves in preparation for the acute motor vehicle trauma that might occur once or twice a year. Many blood centers monitor inventories in the hospitals. The center can then rotate out blood that may be

expiring soon from the hospitals that use less blood to large hospitals and trauma centers that will be able to use the blood before expiration. In the United States in 2006, significantly fewer RBC and whole blood units expired (25% reduction) than in 2004. These included 252,000 allogeneic (nondirected) units, 131,000 autologous units, and 5,000 directed units. The outdate rate (expiration) fell from 3.2% in 2004 to 2.4% in 2006, the lowest outdate rate in recent years.<sup>5</sup> The estimated mean age of RBC units was approximately 17 days at the time of transfusion.<sup>5</sup> Even the small number of allogeneic RBC units that do expire after 42 days are likely to have been available for several patients that ended up not using the blood; the expired blood, therefore, fulfilled its purpose for collection—it was there when it was needed. The low outdate rate suggests that blood centers have become more efficient at delivering the appropriate product when needed. However, when the expiration rate drops too low, then the safety margin of having enough blood at any particular time is reduced. Fewer hospitals reported shortages in 2006 in comparison with 2004 or earlier data. Although the range of days for postponed surgeries was 1 to 120 days, all but two of 117 hospitals reported delays of 1 to 16 days, a much narrower range than in previous years.<sup>5</sup>

## The Collection Process for Source Plasma

The handling of source plasma donors differs in several respects from the handling of whole blood and apheresis donors providing blood products for hospital transfusion. Source plasma is collected by apheresis specifically for further manufacture into biological derivatives, in contrast to “recovered plasma,” which is the plasma remaining after RBC production from whole blood donation. About half of the recovered plasma collected for fractionation comes from Europe. Many European countries have maintained national programs that fractionate recovered plasma from volunteer donors. However, the demand for biological therapies worldwide requires the additional supply from remunerated donors. Almost all source plasma is collected in Austria, Germany, China, and the United States from donors who receive remuneration. In 2005, source plasma accounted for 63.5% of the plasma for further manufacturing worldwide. Approximately half of the 22 million liters collected came from North America with the United States accounting for 98% of the North American total. Of the US total, 8.5 million liters were source plasma and 2.3 million liters were recovered plasma. In the past, there has been a 4 to 1 ratio between source and recovered plasma in US plasma products, and this has generally continued. However, worldwide it is projected that growing automated component collections by apheresis will cause the percentage of source plasma to increase, as blood centers draw less plasma that is not used for direct patient care. In fact from 2000 to 2005 the volume of recovered plasma collected declined by about 14% while the volume collected as source plasma increased by about 4%. About 25% of the total plasma for fractionation comes from Europe, 20% from Asia and the Pacific and small amounts

from the Middle East, North Africa, and Latin America. In the United States in 2006 there were an estimated 12.4 million collections; this number varied between 10.3 and 13.8 million from 2002 to 2006. The number of collection centers during that time decreased from approximately 400 to 315, with annual volume per center increasing to about 39,000 collections annually.<sup>59</sup>

The United States provides about half of the world's source plasma requirements for two reasons: First, under FDA regulations source plasma donors can donate more plasma, more frequently, than is the case in most countries. Second, the United States has a well-developed source plasma industry that has invested in a network of centers that compensate donors for their time and inconvenience.

Source plasma typically is collected in fixed sites without mobile collections. Either the Autopheresis-C (Fenwal, Lake Zurich, IL) or the PCS-2 (Haemonetics, Braintree, MA) is used for nearly all collections. Recruitment is typically by word of mouth, newspapers, radio, and posters. The donor groups are heterogeneous. Sites near college campuses attract student donors. Medium-sized cities and towns are favored over larger metropolitan areas. Payment in the United States varies between \$25 and \$40 for each normal donation of source plasma. More money is paid for donations from immunized donors for hyperimmune plasma (rabies immune plasma, tetanus immune plasma, hepatitis B immune plasma, anti-D plasma, etc) and by those from donors with disease-state antibodies needed for diagnostic manufacture.

In addition to specific national regulations and guidance, in the United States and Europe the International Quality Plasma Program (IQPP) of the Plasma Protein Therapeutics Association (see <http://www.PPTA.org>) offer a certification if quality standards are met (see Chapter 19). This self-regulation includes the following:

- Community-based donors—donors must have a permanent address in the vicinity of the center.
- Qualified donor standard—each donor's plasma is used only after two medical screenings and required viral testings are successful and less than 6 months has elapsed between donations.
- National donor deferral registry—all US donors deferred for viral marker testing are entered into a registry that must be checked before each applicant (ie, not qualified) donor is accepted.
- A viral marker standard that requires centers to keep their viral marker rates below established levels.
- A 60-day inventory hold so units from a donor with a subsequent positive test or disqualifying information can be removed before being pooled.

The community-based donor standard discourages transient individuals from donating, as this population has been associated with an increased incidence of infectious disease markers. Photosensitive nail coloring is applied under the fingernails to ensure the donor donates only at one donor center at a time. A minimum weight of 50 kg (110 lb) must be met. At each donation the hematocrit and total protein are determined from a fingerstick blood sample. The hematocrit must be 38% or greater and protein 6.0 g/dL or greater. The protein is measured using

a refractometer (the only point-of-care device currently available that meets the FDA requirement).

Initially the donor is subjected to an extensive interview similar to that used for blood donors followed by physical examination by a physician. In the United States, a qualified physician substitute may be used (a nurse, paramedic, or other health professional). They are trained to do the physical examination and operate under the guidance of the center medical director. The physical examination consists of external eye, ear, and nose exam, with an examination of the throat with tongue blade and light. Lymph nodes in the neck area are palpated. Auscultation of the back and front of the chest for lung abnormalities is followed by auscultation of the heart. The abdomen is examined for liver and spleen enlargement. A short neurologic and extremity review completes the examination. In some centers a urine dipstick for protein and glucose may be performed. After passing these initial tests, the donor can donate as an applicant donor. The donor must initially and every 4 months pass tests that include serum protein electrophoresis, total protein, and syphilis. The physical examination with full interview is repeated annually, or if the donor has not presented in the past 6 months. An informed consent must be executed on the first donation and again whenever the consent is changed. Collected units are held until the donor has two successful donations without any positive infectious disease markers. If the donor does not return for the second donation, the plasma is considered an "orphan" unit and cannot be used for injectable product.

In the United States the donor donates according to a nomogram: Body weights of 50 to 67.5 kg, 68 to 79 kg, and greater than 79 kg can donate 625 mL, 750 mL, and 800 mL of plasma respectively, net of anticoagulant. Some centers infuse saline at the end of the donation. After a brief rest in the donation chair, the donor collects the remuneration and is thanked for donating. Refreshment and recovery areas are not common in plasma donor centers, although centers provide additional attention to first-time donors. In the United States, donors are allowed to donate no more than twice in 7 days, with at least 2 days between donations. A donor could theoretically donate 104 times per year (65 to 83 L, depending on donor weight).

The Council of Europe recommendations limit the amount of plasma collected per session to 650 mL, net of anticoagulant and no more than 1.5 L per week. There is a 25-L annual limit (or 28.5 L including anticoagulant). Current German national guidelines set in 1999 allow donations of up to 750 mL plasma including anticoagulant twice weekly (or 850 mL if a male donor weighs over 85 kg or a female donor weighs over 90 kg), with an annual limit of 28.5 L. Donors must weigh a minimum of 50 kg and have a hemoglobin level of 12.5 g/dL in females and 13.5 g/dL in males. Immunoglobulin G (IgG) is typically measured at every fifth donation, and total protein every fifth donation must be at least 60 g/L. (The European guidelines state these should be done at suitable intervals but at least annually).

To determine if less restrictive donation standards (closer to those in the US) were safe, 21 German plasma donor centers participated in a study known as SIPLA. They found that donors

weighing greater than 70 kg could donate 850 mL each session up to 60 times per year with appropriate monitoring (up to 51 L total per year). Currently there is an effort to change European requirements to be consistent with these findings.<sup>60,61</sup>

Each donation by a source plasma donor is tested serologically for HIV antibody, HBsAg, and anti-HCV. NAT is performed on each donation for HBV, HCV, and HIV. A positive test results in deferral. This is regardless of results of confirmatory tests that are performed for donor counseling. In addition, NAT tests for hepatitis A and parvovirus are also performed. If positive, the units are discarded but donor status is not affected and there is no counseling.

Because of the importance of developing a history on each donor, donor centers cultivate relationships designed to attract long-term donors who donate often. The average US donor donates four to five times a month and remains in the program for months to years. Some very dedicated donors are given vaccines to provide rabies immune plasma, tetanus immune plasma, hepatitis B, or other immune plasma in order to make specialty immunoglobulins.

Rh-negative donors who are not of childbearing potential can become donors of Rh-immune plasma for manufacture into Rh Immune Globulin. This is done by immunization with carefully "qualified" D-positive red cells. Long-term consistent donation is particularly important for these specialty donors.

There are additional requirements for donors receiving red cell immunizations including physician performance of the initial physical examination and a separate informed consent, physician presence when immunizations are given, and a specific physician approval of the red cells to be injected. A laboratory must be specially licensed to produce immunizing red cells. Meticulous preparation of the red cells for immunization occurs. Red cells from whole blood donors are frozen and stored for over a year. Only when a year's viral marker testing remains negative can the cells be deglycerolized and used to immunize a donor. When the red cells from a specific whole blood donor are first used, they are given to one to three recipients for a year. When viral marker testing is negative throughout the year for those recipients as well as the donor, the cells are then "qualified." Plasma from these donors is used to manufacture Rh Immune Globulin and the pool must contain a sufficient concentration (titer) of antibody to allow acceptable product to be made.

Rates of viral marker test positivity in donors are monitored and IQPP-certified plasma centers are expected to take steps to reduce levels when they rise above predetermined alert levels. Source plasma donors are more likely male, younger, and larger in size than volunteer whole blood donors. In addition, they are more ethnically diverse. Some of these factors act to increase prevalence and incidence of viral diseases. Socioeconomic status and compensation programs might play a role as well. With the push toward nonremunerated donations worldwide, the payment of plasma donors has been criticized. Nevertheless, monetary compensation of source plasma donors has had an enviable safety record for the last 15 years because many additional controls have been put in place. The viral reduction and inactivation

treatment of the final product provide additional safety. Thus, the layers of protection operate differently in source plasma programs than in the volunteer blood programs, but are still highly effective in preventing infectious units from entering plasma pools and in ensuring the safety of the final product.<sup>62</sup>

## Blood Donor Adverse Events

### Donor Reactions

Blood and plasma donor reactions are monitored by collection agencies to ensure that donation does not pose a risk to the donor's health. Adverse responses to donation or reactions can be either 1) acute, ie, immediate or delayed after a single donation or 2) chronic, ie, in response to long-term donation.

Variables affecting donor reactions are listed in Table 62-1. Acute reactions most frequently arise from anxiety about painful venipuncture or susceptibility to the blood volume deficit during or after donation. Increased rates of reactions are seen with increased volumes removed, first-time donation, younger age, weight less than 120 pounds, and female gender. In a single study in which multivariate analysis was used, all of these factors were independent predictors of donor reaction with the strongest odds ratios for first-time donation and female gender.<sup>63</sup>

The most common type of reactions is a vasodepressor reaction, associated with changes in pulse and blood pressure. When mild, they are characterized by pallor, diaphoresis, and nausea. More severe reactions include syncope or movements that resemble seizures, and can include unconsciousness. Recovery can take 45 minutes or longer. These often resemble vasovagal reactions

seen in other situations (lowered blood pressure and slow pulse), and peripheral baroreceptor activity is thought to play a major role.<sup>19,64-67</sup> Syncope-related falls are not uncommon and may cause injuries.

Vasovagal reactions occur in 2% to 5 % of blood donors, with 0.8% to 0.34% of donations progressing to syncope. One study reported serious reactions in 0.6 per 1000 donations. They are less frequently seen with plateletpheresis procedures, presumably because of a slower blood removal and return of red cells.<sup>67</sup> In a 1-year study<sup>68</sup> in an urban blood center, the incidence of syncope was 0.09%. Younger donors and female donors had increased rates of vasovagal reactions. The reaction occurred before donation (1%), during or immediately after donation (26%), at the refreshment table (61%), and offsite (12%), and usually within 1 hour. The five most common clinical findings were pallor (87%), dizziness (67%), diaphoresis (49%), hyperventilation (49%), and nausea (25%). Six percent of all whole blood donors with syncope had emesis and 46% of the reactions included clonic movements, tetany, or twitching, and 5% had incontinence (usually urinary). Blood pressure and pulse reduction (hallmarks of vasovagal reactions) were actually minimal in this large series.<sup>68</sup> Fourteen percent had a traumatic injury as a result of the reaction. Six percent of the donors with reactions visited an emergency room, but none were hospitalized. First-time donors are known to have a higher risk of these reactions than repeat donors. In one study of high school donors<sup>69</sup> it was 9.4% vs 3.6%. Low weight adds to the risk. First-time donors weighing less than 59 kg had a rate of 13.6% and females at that weight had a rate of 16.4%.

Adequate hydration before donation along with nutritional intake is considered an important preventive measure by many. A study of high school donors receiving a 16-ounce drink before donation showed a modest 21% reduction in reactions; the mechanism is thought to be gastric distension increasing sympathetic activation.<sup>35</sup> Other methods of reducing donor reactions such as a muscle tensing technique are promising based on both distraction of the donor and maintenance of blood pressure.<sup>36</sup>

Autologous donation is associated with increased and more severe reactions.<sup>70</sup> Studies in autologous donors have demonstrated decreased cardiac output associated with donation (6.9 to 6.0 L/minute). Saline infusion during and after the donation limited the effect.<sup>70</sup> Studies in the well elderly showed surprising resistance to hemodynamic changes,<sup>19</sup> but postural hypotension can be seen in many donors as a normal consequence of donation. Increased heart rate occurs with loss of blood volume from removal, while a slowing of the heart rate generally indicates a vasovagal reaction that can be psychogenic in origin.<sup>19</sup>

Without more data, current measures to reduce reactions include adequate food and fluid intake before donation, reassurance, and distraction from an attentive staff, immediate attention to symptoms with cold packs, raising the lower extremities or lowering the head to promote blood return, saline replacement for apheresis donors, and a recovery period with snacks for donors before departure. Screening measures to ensure healthy

**Table 62-1.** Factors Associated with Donor Reactions\*

Variables associated with increased donor reactions

- First-time donation
- Greater percentage of blood volume removed
- Admitted anxiety
- History of fainting
- Female gender
- History of prior reactions
- Age less than 30
- Tall/thin habitus
- Greater than 4 hours from last meal
- Mobile blood drive
- Low diastolic blood pressure

Variables associated with decreased donor reactions

- Increased number of prior blood donations
- Greater body surface area; increased height and weight
- Increased donor age
- African ethnicity
- Higher diastolic and systolic blood pressure
- Donation in spring season

\*These factors have been found in some studies to influence donor reaction frequency and severity. These data are primarily from studies of whole blood donation.

donors should lessen the problem. Special attention from staff and longer recovery periods for first-time donors are reasonable steps. In addition to concern for donor well being and risk reduction, attention to the reduction of reactions is key to an adequate supply of blood and plasma. In one study, 59% of donors having no reaction returned to donate within a year (334 days); for donors with mild reactions the return was reduced to 26% and more severe reactions, 14%.<sup>67</sup>

### Phlebotomy

Another set of adverse consequence relates to the needle puncture, including hematoma, thrombosis, infection, and physical damage to anatomic structures, such as the median nerve. A hematoma can result at the venipuncture site. Causes include inadequate pressure on the site after donation, the needle going through the vein such that the pressure applied after the donation does not stop the leakage from the back side of the vein, adjustment of the needle, a bleeding tendency (for example in someone taking aspirin), anatomic variation, and vessel or tissue friability. Hematomas complicate 9% to 16% of donations, according to various surveys, but calls to randomly selected blood donors 3 weeks after donation revealed that 22.7% reported a hematoma.<sup>67</sup> Hematomas often go through a process of widespread extension along fascial planes with various discolorations that can be alarming to the donor. Treatment with ice immediately and periodically for 48 hours after the event will retard the bleeding and promote healing. Heat is often used later, but is only of symptomatic value.

Thrombosis can also develop in the phlebotomized vein and is seen in association with hematoma. In almost all cases, these are superficial venous thromboses often misdiagnosed as deep venous thrombosis in emergency rooms. In rare situations, the clot will travel into the deep venous circulation and anticoagulation or thrombolysis will be needed. A most serious combination is septic thrombophlebitis.

Rarely, a phlebotomy site can become infected, in the presence or absence of coincident hematoma. Cellulitis can develop that requires antibiotic treatment. Red streaks from the site, along with warmth, redness, and tenderness accompanied by lymphadenopathy in the axilla are suggestive. The individual is sometimes febrile. It is speculated that trauma to the vein is more common in frequent apheresis donors; however, these same problems are seen in the clinical laboratory setting after a single venipuncture with a small needle.

Acute reactions can also occur when other structures are punctured by the needle. Arterial puncture is a rare event noted when the blood removed is bright red and pulsating. The procedure must be stopped and firm pressure applied to the site for an extended period to ensure bleeding cessation (10 minutes or longer). A pseudoaneurysm is a rare complication.<sup>67</sup> Very rarely there are complaints of injuries to tendons or muscle.

A more common injury that has been studied in more detail is transection of a nerve, particularly the median nerve. A study at a large blood center<sup>71</sup> identified 66 cases during a 2-year period of 419,000 collections, with an incidence of 1 in 6300.

These generally present with numbness and tingling, excessive or radiating pain, with occasional loss of strength. Of the 56 donors who had follow-up, 22 recovered in less than 3 days, 17 in 4 to 29 days, 13 in 1 to 3 months, two in 3 to 6 months, and two in greater than 6 months. Four of the 56 complained of chronic problems that were mild. Because peripheral nerves can regenerate and heal, total recovery occurs in over 90% but it can take a prolonged time. This is probably an inherent problem that is not related to technique and is best treated with arm slings and reduced use whenever possible.<sup>71</sup>

Another complication is related to citrate toxicity. Citrate is the anticoagulant used in almost all apheresis collections. It is a ubiquitous compound found in human cells. Citrate is metabolized to bicarbonate and thus could cause metabolic alkalosis in excessive quantities or if renal disease is present. It acts as an anticoagulant by combining with calcium, which is needed for clotting to occur. Thus, if citrate is infused the ionized calcium level will fall. This leads to increased parathyroid hormone secretion. This in turn stimulates mobilization of calcium from bone and increases intestinal absorption and renal tubular reabsorption of calcium. Citrate does not cause toxicity in whole blood collections, because it is not reinfused. It has minimal effects in donor plasmapheresis because the citrate is mostly in the retained plasma. It is in plateletpheresis, large-volume leukapheresis, progenitor cell collection, and therapeutic plasma exchange with fresh frozen plasma that adverse sequelae resulting from citrate are most likely to be experienced. Symptoms of hypocalcemia seen are most commonly perioral and/or peripheral paresthesias. Symptoms seen less often include dysgeusia (unusual taste), nausea, lightheadedness, shivering, twitching, and tremors. More discussion of citrate reactions may be found in Chapter 42, about therapeutic plasma exchange.

Cardiac toxicity can also occur with citrate anticoagulation. The reduction in ionized calcium lengthens the plateau phase of myocardial depolarization, prolonging the QT interval. This has not been associated with clinical problems, but depressed myocardial contractility may rarely be seen. Although these rarely rise to the level of clinical concern, occasionally citrate toxicity cannot be differentiated from a cardiac event without a clinical evaluation.<sup>72</sup>

Attentive operators can reduce the citrate reaction rates by looking for early symptoms and slowing the outflow and reinfusion. Women and smaller individuals are more susceptible to reactions and thus warrant more careful observation. As new apheresis devices have been brought to market, citrate loads have been decreased and the symptoms have also been seen less frequently. Increased citrate infusion rates are clearly associated with more severe clinical reactions.<sup>73</sup> Various measures may be used to reduce citrate reactions. Administration of calcium gluconate effectively reduce the reactions, but requires careful monitoring.<sup>74</sup> Use of oral calcium supplements can be effective. For the most part the reactions are mild and well tolerated.

Allergic reactions are less common than others. In the past, reactions were seen to ethylene oxide used to sterilize disposable

sets, but this method of sterilization has been largely abandoned. Latex-related reactions are also possible if the environment is not latex free. Hives and minor reactions can be treated with antihistamines, but life-threatening anaphylactic reactions are treated with epinephrine.

Unusual reactions such as air embolism are theoretically possible during donor apheresis, but not reported using modern technology. This rare complication of apheresis is characterized by dyspnea, tachypnea, cyanosis, tachycardia, and hypotension after a large amount of air (typically over 200 mL) enters the venous circulation of the donor.<sup>75</sup>

With the increasing use of apheresis for blood collection—not only for platelets and progenitor cells, but also for red cells—the safety of the procedures is of concern. An analysis of 249,154 2-unit RBC apheresis collections, 40,870 single-unit RBC apheresis collections, and 90,082 plateletpheresis procedures compared with over one million whole blood collections confirmed the safety of the apheresis procedures.<sup>76</sup> Moderate and severe reactions were seen in 0.47% of whole blood donors, 0.37% of single-unit RBC apheresis donors, 0.15% of 2-unit RBC apheresis collections and plateletpheresis collections. These data confirm the favorable safety profile of standard apheresis procedures for collection of red cells and platelets.<sup>76</sup> Part of the lower reaction rate might be explained by the fact that apheresis donors are more likely to be experienced, regular donors than the whole blood donors. An older multi-institutional study<sup>77</sup> had shown a rate of acute reactions among apheresis donors of 2.18%, which is lower than reports of reactions among whole blood donors (11–21%) but higher than the newer larger study reported above. Differences probably relate both to definitions and fewer reactions with newer technology. The rate is higher among platelet donors (12%) than either plasma (5.9%) or granulocyte donors (9.4%). A single institution study<sup>78</sup> reported in 1999 found an overall reaction rate of 0.81% with two reactions out of 19,736 being serious enough to require hospitalization. Citrate reactions account for some of the differences. They are higher with plateletpheresis and with certain equipment.<sup>75</sup> Procedures collecting 2 RBC units at one time are growing in popularity. Studies have shown that, in donors who meet current selection criteria, the physiologic changes (heart rate, blood pressure, and exercise capacity) are not greater than with a whole blood donation except for a slightly greater decrease in  $\text{VO}_2$  max at 24 hours after donation. Cognitive functions have also been assessed and not found to differ significantly. There is a proportionate increase in marrow erythropoiesis, as expected. Thus, automated red cell collection of 2 units appears to be as safe as whole blood donation. The saline replacement and longer draw time probably provide a protective benefit.<sup>67</sup>

### Long-Term Effects of Donation

Long-term effects of donation are limited to those donors who donate frequently over an extended period. Among whole blood donors, the major concern is iron depletion, leading to anemia. Among plasma donors, the major concern is protein depletion (decreased immunoglobulins).

Over 200 mg of iron is lost with each whole blood donation; thus, screening for an adequate hemoglobin level is part of routine donor assessment. An additional measure to protect donors from iron deficiency is the requirement for an 8-week interval between donations. But these measures are not completely effective and iron depletion is frequent among regular whole blood or red cell apheresis donors. In one study,<sup>79</sup> 6% of first-time female donors were found to be iron deficient, while 22% who gave 2 or more units over a 4-year period had low ferritin levels. Corresponding percentages for first-time and repeat male donors were 0% and 9%, respectively.<sup>79</sup> In another study,<sup>80</sup> 0% and 12% of male and female first-time donors were iron deficient, and 16% of women who gave 2 units/year were iron deficient. Only 2% of males who gave this amount were iron deficient, but 19% of males who donated every 8 weeks were iron deficient. Low ferritin levels were frequently found in menstruating women, and menstruating women taking iron supplements had higher ferritin levels than those who did not. The degree of iron deficiency relates to the frequency of donation.<sup>80</sup> Although postmenopausal women have a lower risk, a study of elderly blood donors showed iron deficiency was frequent in those donating approximately four times per year. Male donors need 14  $\mu\text{g/kg/day}$  from dietary sources for physiologic loss and women require 24  $\mu\text{g/kg/day}$  to make up the additional iron loss from menses. Donation three times a year adds another 25  $\mu\text{g/kg/day}$  to this requirement. Dietary iron alone usually cannot make up this loss.<sup>81</sup>

Studies have shown that iron supplementation of menstruating female blood donors can compensate for the loss and allow this group to contribute to the blood supply.<sup>80</sup> But many blood donation organizations have not been willing to institute such a practice, because iron may be used inappropriately.<sup>82</sup> Complicating the problem have been reports of reduced cardiovascular mortality in individuals who are iron deficient, suggesting that this iron depletion might be beneficial to the health of the donor. Countering this are data that iron depletion alone affects mitochondrial metabolism, resulting in cognitive and physiologic impairment (fatigue, decreased work productivity, decreased physical endurance). Restless leg syndrome has also been found with iron deficiency. Iron deficiency anemia results in a clinical condition associated with symptoms when sufficiently severe, and this may be problematic in those 50 years of age and older as it might mask blood loss caused by gastrointestinal malignancy. The current whole blood collection paradigm has a hemoglobin cut-off slightly above the low end of normal in women and below the low end of normal in men.<sup>83,84</sup>

Risks of iron supplementation include masking of bleeding from occult gastrointestinal cancer in an older person, accidental poisoning in children, aggravation of hemochromatosis (if long term), gastrointestinal side effects, interaction with foods and other drugs, rare allergic reactions, and possible aggravation of atherosclerotic tendencies. The positive side would be to allow more women to donate. Newman<sup>83</sup> has proposed introducing short-term iron therapy after donation and simultaneously reducing the hemoglobin acceptance standard. This is particularly important for women of African ethnicity who have lower

normal hemoglobin than women of European ancestry. At the same time, Newman believes the standard should be increased for males, so that they are not accepted when they meet the definition of clinical anemia. These recent recommendations have not yet changed practice. Therefore, frequent donation may lead to iron deficiency and iron deficiency anemia. Many donors will leave the donor pool after being deferred for a low hematocrit or on instruction from their physician who diagnoses the problem.<sup>83,84</sup>

In platelet- and plasmapheresis donors, red cell removal is minimal so that neither iron depletion nor anemia is a problem. The hemoglobin check is simply to ensure the donor is in good health. Plateletpheresis donors at current frequencies do not face issues with either platelet or plasma protein changes.<sup>75</sup>

In contrast, serial plasmapheresis donors are known to be affected by the plasma protein removal at each donation and may not fully recover between donations. Dedicated frequent donors typically have lower serum protein, globulin, and IgG levels than nondonors. Albumin levels are less significantly affected and CD19+ B cells increase and suppressor T (CD8/CD11b) cells and natural killer cells are reduced.<sup>85</sup> In one study, 20% of normal source plasma donors and 42% of primarily female anti-D specialty donors who were very committed to the twice weekly donation had IgG levels below the normal range.<sup>85</sup> However, no clinical consequences were found in an industry-supported study<sup>86</sup> of donors who dropped out of plasmapheresis programs. Medical problems were not associated with their decision to end donation nor were they found on follow-up. Serum protein levels were higher than they had been when the donors were in the program, indicating the ability of the proteins to regenerate.<sup>86</sup>

The current regulations for protection of serial plasmapheresis donors in the United States require measurement of total serum protein at each donation and a protein electrophoresis upon first donation and at 4-month intervals. In German centers, IgG levels are measured at regular intervals instead of the protein electrophoresis. When donors fall below acceptable levels, they cannot donate and must have a repeat measurement in the acceptable range before resuming donations. General experience is that a rest of 1 or 2 weeks from donation permits the proteins to rise again to acceptable levels. One issue is that the refractometer (point of care) measurements of plasma protein give less accurate than the laboratory measurements, but no other method for measurement at each donation is currently available.<sup>85</sup> In addition, the protein electrophoresis measurement does not assess for specific IgG levels that could be reduced affecting immune surveillance. However, in the absence of any associated clinical problems in the donor population, the current FDA donor eligibility requirements have not been changed. In some other countries, lower acceptable frequencies and maximum annual total liters drawn by plasmapheresis are felt to provide additional protection.

Immune suppression is not a problem in whole blood donors or plateletpheresis donors. Thrombocytopenia has not proven to be an issue with long-term platelet donation. Altered bone metabolism and osteopenia has been noted in long-term

platelet donors because of citrate, but these findings are preliminary and not currently considered an issue. Long-term granulocyte or progenitor cell donation could result in lasting effects from the steroids and growth factors used to stimulate increased cells for collection, but this has not been a clinical problem.<sup>75</sup>

In summary, the adverse consequences of donation are generally well understood such that donors can be adequately protected. Efforts to reduce reactions will be rewarded because donors who have reactions or suffer long-term consequences such as anemia or reduced serum protein are less likely to return.<sup>87</sup> Current standards and practices appear adequate to protect the donor's health and safety, but continued vigilance is appropriate, just as it is with transfusion risk.

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## Conclusion

The recruitment and screening of donors and the collection, processing, and testing of blood has developed dramatically over the past half-century. It is a highly regulated and technology-rich field. Much of the blood worldwide is collected by blood collection agencies that may differ greatly in structure and organization. Systems that are very different may still be successful in collecting and delivering a beneficial, life-saving product to patients when they need it. At the same time, the disparity between resources used in highly industrialized countries and other countries for procurement of safe blood is perhaps greater than in any other area of health care.

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## Disclaimer

The authors have disclosed no conflicts of interest.

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Blood transfusions present a paradox. They are believed to be safer than ever before. Yet, there has been no reduction in the number of legal controversies relating to blood. In part, this is because both the government and the general public have a low tolerance for risk in this area. The Food and Drug Administration (FDA) has mandated deferrals and tests whose utility, in many instances, has been questioned. For example, according to one academic estimate, nucleic acid amplification testing (NAT) costs between \$4.7 and \$11.2 million per quality-adjusted life-year (QALY) gained.<sup>1</sup> Indeed, the academic literature has acknowledged that blood safety measures are judged by a different standard than most health-care interventions.<sup>2</sup> The FDA may simply be mirroring public attitudes, as surveys have reported that many people erroneously believe AIDS can be contracted from donating blood.<sup>3,4</sup>

Another consideration is scientific and technological progress. Advances in cord blood and tissue transplantation, for example, have raised new ethical questions. Additionally, privacy concerns have taken on added weight in our society.

Given this delicate legal landscape, legal issues continue to surface in transfusion medicine. This chapter focuses on the following five areas:

- Classic blood transfusion injury claims.
- Donor injury claims.
- Tissue.
- Cord blood.
- Patient privacy.

## Blood Transfusion Injury Claims

Although technological advances have largely eliminated the most serious recognized infectious disease threats to the blood supply, such as human immunodeficiency virus (HIV) and hepatitis

C virus (HCV), lawsuits are still filed in the rare instances where transfusion recipients develop blood-borne diseases or suffer adverse reactions.

## Blood Shield Laws

Forty-nine of the 50 American state legislatures have enacted blood shield laws. Each of these laws deems blood to be a “service” rather than a “product.” As a result, a transfusion recipient generally must prove negligent “service” on the part of the blood center, the hospital, or the ordering physician—not merely that he or she received a defective “product.” As a practical matter, these blood shield laws have eliminated strict liability claims or breach of implied warranty claims against blood providers. In addition, even though New Jersey, the District of Columbia, and Puerto Rico lack formal blood shield laws, each of these jurisdictions has court precedents requiring negligence to be proved when a claim is brought against a blood supplier.<sup>5</sup> Negligence is thus the dominant legal standard.

The precise terms of blood shield laws vary considerably from state to state. For example, a few blood shield laws cover blood only, and not tissue.<sup>6</sup> Thus, while the general effect of blood shield laws is to eliminate non-fault-based claims, the exact wording of the state statute should be reviewed in the event of litigation.

State law should be consulted for two additional reasons. First, the transfusion injury claim may be governed by a health-care liability reform measure that has been adopted in that jurisdiction. Typically, such laws are drafted to benefit all “health-care providers.” In several instances, state legislatures have expressly included blood centers in the definition of “health-care provider”<sup>7</sup>—or courts have found that they should be implicitly included.<sup>8</sup> Second, special charitable immunity protections may apply to nonprofit hospitals and blood centers in that state.<sup>9</sup>

## Proving Negligence

Negligence typically requires the plaintiff to prove all of the following:

- A duty exists running from the defendant to the plaintiff.
- The defendant failed to meet the relevant standard of care.

- The defendant's failure to meet that standard of care proximately caused injury to the plaintiff.
- Damages were suffered by the plaintiff.

In transfusion injury litigation, "duty" is frequently at issue. For example, a number of claims against hospitals have been rejected on the ground that the attending physician, not the hospital, had the duty to warn about the risks of blood transfusion.<sup>10</sup> Conversely, some cases hold the transfusing hospital liable for the negligent actions of their physicians. "Duty" generally focuses on the relationship of the parties and whether the law recognizes an obligation on the part of the defendant to protect the plaintiff from the type of injury that occurred. Historically, a mere "bystander" does not have a legal duty to render aid to an injured party. However, as illustrated by one Ohio court, a community-based blood bank may owe a duty to provide blood to a person in a known life-and-death situation, even though it did not serve that hospital. The court emphasized the unique circumstances of the case.<sup>11</sup>

More frequently, liability depends upon how the issues of "standard of care" and "causation" are resolved—specifically on whether the defendant fell below the standard of care in some respect (such as by not deferring a specific donor, not rejecting a particular unit, or not using a certain assay) and on whether the failure to meet the standard of care *probably* caused the plaintiff's illness.

At the theoretical level, negligence is supposed to involve a cost-benefit calculation where the overall costs of a precaution are taken into account. Only if anticipated benefits of a safety measure exceed anticipated costs is the defendant deemed to have acted "negligently" by not implementing that measure. As one well-known judge put it: "[I]f the probability be called P; the injury, L; and the burden, B; liability depends upon whether B is less than L multiplied by P: ie, whether  $B < PL$ ."<sup>12</sup> However, public tolerance for risk in the blood area appears to be very low. As a practical matter, a blood center or hospital that does not follow the "state of the art" may be at legal risk if a transfusion-related injury occurs.

Additionally, in recent years, plaintiffs and their attorneys have become increasingly creative in fashioning theories of negligence. One such theory is that the transfusion recipient should have been given the opportunity to arrange for directed or autologous donations.<sup>13</sup> In light of the number of cases that have arisen in this area, it makes sense from a legal standpoint for a health-care provider to offer this opportunity when it is practical to do so and when the standard of care in the relevant community follows the practice, even though the value of both autologous and directed donations has been challenged. In another fairly recent case, a jury awarded \$6 million to the widow of a transfusion recipient who had allegedly wanted only autologous transfusions, although this verdict was reversed on appeal.<sup>14</sup>

Another example of legal creativity and innovation was a lawsuit brought against a Florida blood center when a young patient died after contracting West Nile virus (WNV) from a transfusion. No test available at the time would have detected the presence of the virus in the donor's blood, and the donor was completely asymptomatic. Nevertheless, the plaintiff maintained that the donor, an American citizen residing in Puerto Rico, did not speak English well enough to have been capable of responding accurately to donor

history questions. Although the plaintiff never showed that the blood center's alleged negligence (ie, failing to screen out this donor based on his allegedly limited understanding of English) was more than fortuitously connected to the harm that actually occurred (because even if the donor had understood English perfectly, he still would have been allowed to donate and would have passed along WNV), the trial court allowed the case to proceed. The jury then awarded over \$8 million.<sup>15</sup> In October 2007, an appellate court reversed this verdict, ruling that the claim was covered by a recent amendment to the Florida health-care liability reform legislation, and that the plaintiff had failed to comply with its provisions by not providing a presuit notice to the blood bank.<sup>16</sup> At this writing, the case is being considered by the Florida Supreme Court.

Bacterial contamination claims also appear to be increasing in transfusion litigation, because it can be more readily detected than in the past. Typically, the plaintiff names both the blood collector and the hospital when bringing a lawsuit, arguing that the delivery and transfusion of a contaminated unit could have been prevented at either stage.<sup>17</sup>

## Defining the Standard of Care

Because of the public's low tolerance for risk in blood transfusions, the key issue in a transfusion injury case often is whether the plaintiff can convince a judge to allow his or her case to go to trial. Once a case proceeds to a jury trial, the blood provider risks an adverse outcome. Thus, a defendant's goal often is to defeat the claim *before* trial through the pretrial device of "summary judgment." Summary judgment can be granted by the judge when he or she believes that the case is not supported by sufficient evidence to warrant jury submission.

In this regard, several questions need to be considered:

- Does the plaintiff need to establish a breach of the standard of care through testimony by a qualified expert?
- If so, who is considered to be a "qualified" expert?
- Regardless of what an expert says, does the blood provider have a complete defense if its conduct met prevailing industry standards?

These are critical questions because 1) the absence of expert testimony, 2) the absence of "qualified" expert testimony, and 3) the satisfaction of prevailing professional/industry standards are all potential defenses that may allow a claim to be disposed of on summary judgment.

## The Need for Expert Testimony

The prevailing view is that blood centers, like physicians, are entitled to be judged according to a professional standard of care. As a practical matter, this means that the plaintiff often must have an expert to support his or her lawsuit because a layperson cannot testify about a professional standard of care. As one Ohio court has said, "Because the operations and tasks of hospitals and blood banks are not within the purview of common knowledge, plaintiffs' negligence claims... require expert testimony."<sup>18</sup> A Nevada court made a similar point, acknowledging the clear and growing consensus of jurisdictions that view the production and safeguarding of the nation's blood supply as a professional activity,

entitled to a professional standard of care: “We are convinced that determinations concerning the testing of donated blood and the exclusion of categories of donors are better suited to professionally trained members of the industry rather than laypersons. Such determinations require professional expertise in adopting procedures necessary for securing healthy blood and blood products without dangerously impacting the availability of adequate blood supplies.”<sup>19</sup> Thus, without supporting expert testimony, the typical transfusion-related lawsuit will not be allowed to go forward.

### The Expert’s Qualifications and Opinions

Over the last 15 years, courts have subjected experts’ qualifications and opinions to much tighter scrutiny. In 1993, the US Supreme Court decided *Daubert v. Merrell Dow Pharmaceuticals, Inc.*,<sup>20</sup> which has ushered in an era of greater court skepticism about experts. In that famous case, involving a challenge to expert testimony on whether the morning sickness drug Bendectin caused birth defects, the Supreme Court adopted a four-factor test on whether expert testimony should be admitted: 1) whether the theory/technique issue can be tested; 2) whether it has been subjected to peer review and publication; 3) the known or potential rate of error; and 4) whether it has been generally accepted within the scientific community. Although these factors are not absolute prerequisites, *Daubert* on the whole has encouraged trial courts to look more closely at whether the scientific community would accept a particular expert’s testimony before allowing a jury to hear it.

Although *Daubert* is technically binding only on federal, not state, courts, it has influenced state court attitudes toward experts as well. Furthermore, since the early 1990s, a large percentage of blood transfusion claims have been litigated in federal court, because the Supreme Court ruled in 1992 that the Red Cross defendant in transfusion-related cases could compel any case in which it was named as a defendant to be tried in federal court.<sup>21</sup>

A Colorado case illustrates this trend toward increased judicial scrutiny of experts. There, a patient received a 1993 transfusion that was HIV positive because the donor had given blood during the so-called “window period.” The plaintiff’s expert argued that the blood center should have followed a procedure of freezing and quarantining blood donations for at least 6 months, and then retesting its donors, before releasing the blood. The court found that this approach was not sufficiently accepted within the scientific community and refused to allow the case to proceed to trial.<sup>22</sup> In another application of the *Daubert* case, the Texas Supreme Court decided that a trial court acted properly in excluding the plaintiffs’ expert and dismissing their case when it discovered the expert had no medical degree and had obtained his PhD by correspondence course from an unaccredited university.<sup>23</sup> In a Delaware case, a trial judge was reversed for questioning the credibility of a plaintiff’s expert excessively in the presence of the jury, although here again the court affirmed that the *Daubert* prerequisites apply to expert testimony.<sup>24</sup>

### The Relevance of Prevailing Standards

It is well-settled that governmental requirements alone generally do not define the standard of care in a blood transfusion

case. Thus, mere compliance with current statutory/regulatory requirements is typically not a complete defense for a hospital or center. At the same time, failing to follow FDA regulations can establish a breach of the standard of care. Thus, legal requirements are a kind of one-way street: they can serve as a basis for liability but do not necessarily exonerate a blood provider.<sup>25</sup>

Less clear is the weight to be accorded industry standards in a blood transfusion case. In a medical malpractice case, the plaintiff typically must show that the defendant physician failed to meet the prevailing standards of his or her profession. Without proof that the defendant’s conduct fell below the applicable standard of care in his or her community, the plaintiff’s lawsuit will usually fail.

Applying that analogy to blood collection and transfusion, a number of courts have decided that the plaintiff cannot pursue his or her case unless there is evidence that the defendant fell below the *prevailing* standard of care. Thus, for example, in Alabama, California, Ohio, and South Carolina, courts have indicated the blood bank can defend a case completely by showing it met the prevailing standard of care.<sup>26</sup> Elsewhere, as for example in Colorado, the District of Columbia, and Illinois, the prevailing standard is not a complete defense: The plaintiff is entitled to demonstrate that the industry standard was deficient.<sup>27</sup>

This debate is likely to continue into the future. However, where a state has passed a medical liability reform statute that includes blood establishments, as is the case in a number of jurisdictions, the blood bank ought to be able to argue persuasively that it should be treated no differently than a traditional health-care provider and, therefore, that meeting the prevailing industry standard is sufficient. In Arizona, Florida, and Indiana, for example, the statutory definition of “health-care provider” in their medical liability reform laws expressly includes blood banks.<sup>28</sup>

Even when a blood provider is legally entitled to rely on the prevailing industry standard, there may be disagreement as to what that standard is. One difficult area is the situation where a precaution is available but has not been generally adopted. Has it become an industry standard or not? As the costs of new assays and new procedures increase, this situation may become more common. For example, blood centers have followed divergent practices in leukocyte reduction, in testing for Chagas’ disease, and in responding to transfusion-related acute lung injury (TRALI). Where some blood collectors or transfusion centers follow a procedure, a center that does not follow the procedure may be at legal risk *if* the failure to follow the practice can be proved to result in injury. For example, a TRALI claim that failed, based on the consensus in the blood banking community as of the date of the transfusion,<sup>29</sup> might succeed at a future time.

### Causation

As noted above, the plaintiff in a transfusion injury case also must prove causation, namely, that the violation of the standard of care was responsible for his or her injuries. The more cutting-edge theories, such as failure to perform surrogate or single-donor (as opposed to pooled) testing, can pose difficult causation questions. For example, in one HCV case involving surrogate testing, the court specifically held that a lawsuit could

not proceed to trial based on insufficient evidence of causation. The court emphasized that the plaintiff had not presented evidence that the unperformed alanine aminotransferase test “more likely than not” would have screened out the donor carrying the hepatitis C virus.<sup>30</sup> This “more likely than not” rule is reflective of traditional tort doctrine that a plaintiff must prove each element of his or her case to a greater than 50-50 probability.

### **Lack of Informed Consent and Related Theories**

Although more sophisticated and up-to-date consent processes and forms have made it more difficult to pursue transfusion-related claims based on the patient’s lack of consent, these claims continue to be asserted in some circumstances. For example, if the recipient consent process does not include an explanation of a material risk or alternatives, defined as a risk that a “reasonable” person would have considered significant, this may give rise to a claim of medical negligence. Transfusion recipients or their representatives have alleged in some cases that the consent process was incomplete because it did not mention alternatives such as directed donations.<sup>31</sup> Or, the family may contend that either no transfusion or a transfusion from directed donations had been promised or requested, giving rise potentially to a breach of contract claim where the promise was not kept.<sup>32</sup>

### **Standard-Setting Claims**

Plaintiffs have also—on a few occasions and with mixed success—sued the entities that promulgate blood collection and transfusion standards. Thus, in a few jurisdictions, courts have allowed claims against the AABB to proceed for “negligent promulgation of standards.”<sup>33</sup> Those claims have not been uniformly permitted, however, causing a chilling effect on standard-setting organizations.<sup>34</sup> Allowance of those claims seems to go against the grain of American tort law, which normally requires the defendant to have been involved in delivering some product or service used by the plaintiff. Otherwise, the defendant is typically said to have had no duty to the plaintiff.

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## **Donor Injury Claims**

Donor injury claims appear to be increasing. The most prevalent donor injury claims fall into two categories: 1) needle-related injuries<sup>35</sup> and 2) donor reaction claims—ie, allegations that the donor fainted or fell after the donation process because of the loss of blood.<sup>36</sup>

In most jurisdictions, for a plaintiff to bring a successful donor injury claim, he or she must prove not merely that the defendant’s conduct caused injury, but also that the defendant’s conduct fell below the applicable standard of care. If, for example, a donor suffers a nerve injury that was simply a rare, unfortunate, and unavoidable accident, then the blood center should not be held liable.<sup>37</sup> One court recently concluded that a plasmapheresis donor who alleged the injection of blood components into her arm tissue caused reflex sympathetic dystrophy could not rely on the legal doctrine of res

ipsa loquitur.<sup>38</sup> Res ipsa loquitur (“the thing speaks for itself”) is a legal shortcut that allows a plaintiff to forgo the need for direct proof of causation and a breach of the standard of care, based on a presumption that this kind of injury would have not occurred in the absence of negligence. Although res ipsa loquitur has been invoked successfully in donor injury cases,<sup>39</sup> the recent trend appears to be against its use and to require independent proof of negligence.

It is not entirely clear why donor injury claims seem to be increasing. Blood centers generally have sophisticated risk-management programs. Recognizing that a volunteer blood donor makes for a very sympathetic claimant, centers have generally followed the prudent policy that the “donor is always right.” The frequency of donor injury claims may reflect the fact that, from an attorney’s standpoint, these claims are relatively easy and inexpensive to bring. Unlike the case with a transfusion claim, the plaintiff’s attorney does not have to overcome difficult questions of tracing causation and what level of testing was considered the relevant standard of care. Also, in some instances, donor injury claims have been found to fall outside the scope of medical malpractice reform laws, which require plaintiffs to submit their claims to medical liability review panels and to support their claims with expert medical testimony.<sup>40</sup>

Thus, while the plaintiff must prove negligence in a donor injury case, it may be possible to establish negligence without even the need for expert testimony—or with only limited expert testimony. For example, a plaintiff may allege that he or she should have been escorted from the donation table and was not,<sup>41</sup> or that the defendant was negligent in hiring and training a particular phlebotomist.<sup>42</sup>

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## **Legal Issues Relating to Cord Blood**

The high quality of hematopoietic stem cells in umbilical cord blood has led to an increase in the number of cord blood transplants since the first transplant in 1988<sup>43</sup> to over 1,964 in 2007.<sup>44</sup> Medical techniques to harvest cord blood have improved and physicians are steadily expanding cord blood use to combat a broader variety of diseases.<sup>45</sup> Governmental efforts have recognized the potential of cord blood and have taken steps to enhance its usage. Separately, the promise of cellular therapy derived from cell lines found in cord blood has resulted in a growing business of storing cord blood for potential self use in the future. As a result, both in the United States and internationally, health-care professionals, governmental units, academic institutions, for-profit and not-for-profit corporations are involved in activities that give rise to a variety of new and complex legal issues. Those involved with cord blood transplants must thoughtfully address changing legislation and legal issues or run the risk of serious consequences.

### **Informed Consent**

As with any medical procedure, informed consent is crucial. In the context of cord blood collection, consent is required when

the blood is collected and stored for potential use in future transplantation.<sup>46</sup> The American Academy of Pediatrics (AAP) has published recommendations for physicians consulted by prospective parents about cord blood banking and guidance for organizations involved in cord blood banking.<sup>47</sup> These recommendations place the burden of obtaining proper consent and educating prospective parents about the cord blood collection process on the physician.<sup>48</sup> The consent process has even been written into federal law in the Stem Cell Therapeutic and Research Act of 2005 (The Federal Act) for cord blood collected under the program.<sup>49</sup> Several states have placed the burden of obtaining consent largely upon the hospitals and physicians.<sup>50</sup>

Materials provided to the mother should contain at a minimum a family medical history questionnaire, information pertaining to what tests are to be performed on the cord blood, an explanation of how the parents will be informed should the test results be abnormal,<sup>46,51</sup> and the range of banking opportunities.<sup>49,50</sup> The consent must be given in writing and before labor begins.<sup>46,47,52</sup> In the event human research is involved, an institutional review board (IRB) must approve the consent form and approve all research protocols.<sup>53</sup> Few cord blood banks obtain both maternal and paternal consent because of the difficulty in obtaining consent within the short time available for successful processing. A report by the Institute of Medicine (IOM) recommended that a plan to address paternal objection to cord blood donation be developed.<sup>52</sup>

### The Medical Procedure

A sufficient quantity of cord blood is critical to a successful collection and future transplantation. Uniformly in the public banking arena and often in the private banking arena, unless the quantity collected meets certain standards,<sup>54</sup> the collection will be deemed inadequate for future use. An interesting and productive effort to address this quantity issue has been the increase in the use of multiple cord blood units for a single transplant procedure. Several medical centers are performing clinical trials to evaluate the use of multiple cord units to enhance engraftment.<sup>55</sup>

After birth, umbilical cord blood is collected by an obstetrician, nurse-midwife, or designated collector.<sup>56</sup> The collection procedure involves clamping the umbilical cord soon after the birth of the child.<sup>57</sup>

There is a risk to the child and thus potential legal exposure to the physician and possibly others if the clamping of the umbilical cord is not timed appropriately. Early cord clamping has been associated with anemia in the child and has led some practitioners to delay clamping for several minutes. Other practitioners believe that delayed umbilical cord clamping may be harmful because of increased blood volume to the child, which may increase the likelihood of future medical complications.<sup>58</sup> Earlier clamping of the umbilical cord increases the proportion of infant blood retained in the placenta and thus the volume of cord blood available for banking. The results of a 2007 study indicated that delaying clamping of the umbilical cord for at least 2 minutes after birth consistently improved both short- and long-term hematologic and iron status of infants.<sup>58</sup>

The AAP and the IOM have both indicated that the cord blood collection program should not alter routine practice for the timing of umbilical cord clamping.<sup>47,56</sup> Furthermore, in the event of a complicated delivery, cord blood collections should not be performed.<sup>47,56</sup>

### Private and Public Banks

There are both “private” or “family” banks (referred to here as private banks), and “public” cord blood banks. Private cord blood banks store cord blood for the future use of the donor or the donor’s family.<sup>56,57</sup> The private banks generally charge a fee for the collection, processing, and storage of cord blood. A contract or service agreement is entered into between the private cord blood bank and the family. Numerous states have enacted legislation specifically recognizing the importance of regulating cord blood donation programs and providing standard, objective information to explain the differences between public and private donation.<sup>50</sup> Recommendations by the AAP discourage private cord blood donation as “biological insurance” for a child.<sup>47</sup> Cord blood can rarely be used for the child from whom it was obtained.<sup>59</sup> A majority of the diseases that have been successfully treated by stem cell transplantation require the use of stem cells obtained from a different individual. The range of estimates that a child will need his or her own privately stored cord blood cells is from 1 in 1000 to 1 in 200,000.<sup>59</sup> Private cord blood banking is recommended for families with an older child with a genetic immune deficiency or similar disorder that may be successfully treated by hematopoietic stem cell transplantation (HSCT).<sup>48</sup>

Public cord blood banks store cord blood for public usage by unrelated donors.<sup>47</sup> There are an estimated 175,000 to 200,000 units of cord blood in public banks.<sup>56</sup> Public banks do not charge the donating family or hospitals a fee for processing, testing, or storing donated cord blood. A fee of approximately \$25,000 is typically charged by the cord blood bank to the transplant center when a unit is provided for transplantation. This fee is usually passed on to the patient and is frequently covered by health insurance.<sup>56</sup> As part of the consent process, physicians are encouraged to inform prospective parents that cord blood donated to a public bank will be available to any patient in need of transplant and will likely not be accessible for the family’s private use.<sup>47</sup>

### Standards and Accreditation of Banks

Increasingly, cord blood banks are accredited in accordance with national standards developed by the AABB or the Foundation for the Accreditation of Cellular Therapy (FACT). Additional standards are maintained by the National Marrow Donor Program (NMDP). All cord blood banks collecting cells for HSCT are required to register with the US FDA.

### Privacy and Cord Blood

In addition to the traditional privacy issues involved in blood-related procedures, the use of cord blood comes with special

privacy requirements. In defining what is a qualified cord blood bank for the purposes of receiving federal funds, the law states that such a bank must have “established a system of strict confidentiality to protect the identity and privacy of patients and donors in accordance with existing Federal and State law; . . . and . . . a system to confidentially maintain linkage between a cord blood unit and a maternal donor.”<sup>49</sup> In regard to patient privacy and cord blood donor records, the IOM recommends that “[s]ecure links between the medical records of the donor and the banked cord blood unit be established to ensure the safety of transplantable products and the patients receiving transplants.”<sup>52</sup>

## Selecting the Cord Blood Unit

The Federal Act, and actions of the US Department of Health and Human Services (DHHS), have resulted in significant efforts to facilitate more cord blood collection and usage. Pursuant to contracts with DHHS, the NMDP maintains a registry of publicly available cord blood units. This registry is to function as a single point of access for patients seeking a transplant.<sup>49,60</sup> Several states have also enacted laws regarding the collection of cord blood that have affected the selection process.<sup>50</sup>

Cord blood is also available directly from some cord blood banks or through registries established by other countries or groups of cord blood banks such as the International NetCord Foundation or AsiaCord, each having created a network that promotes access to units stored by its members.

## Storing, Shipping, and Selling

When cord blood has been properly collected and tested, it must be frozen and stored by a cord blood bank. These actions give rise to potential liability issues. A cord blood bank compensated for its storage services will in all likelihood be labeled a “bailee” under court-defined common law. A bailee has the legal duty to exercise “ordinary care” when dealing with the property of the bailor. Although courts have addressed primarily the duty of ordinary care as applied to private blood banks, a similar duty extends to cord blood banks. A violation of the duty of ordinary care occurs when the bailee “[does] something blood bankers of ordinary care, skill, and diligence would not have done under similar circumstances.”<sup>61</sup> The meaning of ordinary care may also be derived from approved industry customs or practices.<sup>57</sup> In addition, the FDA has promulgated regulations governing storage, which require all blood and tissue manufacturers, including cord blood storage companies, to follow current good tissue practice (cGTP) regulations.<sup>62</sup> The FDA has indicated its intent to require public cord blood banks to meet current good manufacturing practice (cGMP) regulations as well.<sup>63</sup>

Labeling is also regulated by the FDA<sup>64</sup> and the labeling process is closely scrutinized by accreditation groups. In the unlikely event a mislabeled unit got through the required multiple check system and was actually used in a transplant, serious liability issues by would face all involved.

The applicability of blood shield statutes,<sup>65</sup> express and implied warranties of fitness for a particular purpose, and merchantabil-

ity of cord blood under the Uniform Commercial Code (UCC) also need to be considered.<sup>66</sup> In the sale and transfer of significant quantities of cord blood such as on the closing or merger of cord blood banks, the validity of security interests under the UCC should be reviewed.<sup>67</sup> Standard forms (which were probably designed before cord blood was ever sold or shipped) should be carefully reviewed by counsel before being used in a cord blood transaction.

The act of shipping cord blood from a cord blood bank to a transplant center or other location may give rise to additional novel legal issues. If shipping is international, the applicability of custom inspections and air carrier regulations may come into play. Efforts are under way to make these international requirements uniform and make cord blood readily available for transplantation anywhere in the world. However, the international nature of many transplant procedures still raises potential legal issues for the various contracts, agreements, bills of lading, etc that are frequently involved. Cord blood bank licensing requirements also differ from country to country.

## Ownership

The question of “ownership” of cord blood presents another area that has no clear legal answer. Both mother and father may logically claim some interest in the collection. It has also been suggested that a newborn has not legally abandoned his or her property rights to his or her own cord blood.<sup>68</sup> This raises the potential question of whether an individual, upon reaching the age of majority, acquires an interest in his or her own cord blood being held in private storage. Moreover, private banks may also claim an ownership interest in a unit if an individual whose cord blood is banked should die or default on collection or storage fees.<sup>69</sup> In the case of public banks, the donation is quite clearly made by the parents for use by the public; therefore, a strong argument can be made that neither the family nor the child have a unique interest in the unit. As noted below, the terms of the relevant consent documents and the extent of disclosure of information to the parents are likely to play a factor if and when courts face this issue.

No cases have yet addressed directly the property rights of umbilical cord blood, but cases related to human cells, tissue, and organs provide some guidance. In the context of analyzing the validity of a patent and whether private cord blood banking was a service, the US Court of Appeals for the Federal Circuit found that cord blood remained property of the family for the period in which the defendant private cord blood bank stored the unit.<sup>70</sup> The landmark case of *Moore v. Regents of the University of California*<sup>71</sup> held that a person does not possess property rights in his or her own cells or tissue or any profit made therefrom. This case involved a physician and other defendants obtaining patent rights to the creation of a cell line through the use of the plaintiff’s blood and marrow. Based on the reasoning of this case, at least one commentator has written that “*Moore* should not control in identifying property rights in privately stored cord blood.”<sup>72</sup> Certain other case law supports the notion that the storage of human cells creates a property right in the owner of the cord blood unit.<sup>73</sup>

## The Future

Millions of dollars are being invested in research and infrastructure to assist patients in need of a cord blood transplant and to provide a return on these investments in both human and dollar terms. It is difficult to draft appropriate and workable documents to obtain consent, protect privacy, and comply with regulations and accreditation standards. Unresolved issues must be managed. Science, medicine, and law all intersect, each with their own methods and time lines. Cord blood transplantation presents a challenging opportunity for professionals to creatively combine scientific, medical, ethical, and legal concepts to help save lives throughout the world.

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## Legal Issues in Tissue Banking

Medical science has long recognized the clinical and humanitarian value of donating and transplanting conventional human tissues—musculoskeletal, cardiovascular, and skin—to enhance and save lives. This history what has become known as “tissue banking,” traces the roots to 1949 and the establishment of the first tissue bank, the United States Navy Tissue Bank. The Navy’s program developed many of the fundamental clinical standards, practices, methods and techniques that are still in use.<sup>74</sup>

The Navy Tissue Bank led to the creation of the American Association of Tissue Banks (AATB) in 1976. More than 30 years later, the importance, widespread application, and growth of tissue transplantation are evidenced by the 103 tissue banks accredited by the AATB. This exponential growth in tissue banking and tissue transplantation has been fueled by the development of precision musculoskeletal grafts and novel tissue-based products for an ever-increasing assortment of clinical applications to treat a continually expanding list of medical conditions. Today, AATB-accredited facilities retrieve, process, store, and distribute annually more than two million tissue grafts from 30,000 to 40,000 donors in the United States (AATB, unpublished data).

## Legal and Regulatory Oversight

The legal oversight of human tissue is governed by statutes and regulations at both the federal and state levels. The regulations are supplemented by detailed industry standards and private accreditation programs. Established case law is sparse and focuses on issues of ownership.

### Federal Laws

The principal federal regulatory jurisdiction of tissue lies with the FDA and is based on Section 361 of the Public Health Service (PHS) Act.<sup>75</sup> The PHS Act authorizes the FDA “to make and enforce such regulations as . . . are necessary to prevent the introduction, transmission, or spread of communicable diseases” and “to provide for such inspection. . . as in (the agency’s) judgment may be necessary.” A tissue bank that refuses to allow FDA inspection may be prosecuted.<sup>76</sup>

Another major federal statute that touches tissue banks is Title III of the National Organ Transplant Act (NOTA), Public

Law 98-507, approved October 19, 1984, and amended in 1988 and 1990.<sup>77</sup> This law forbids the sale for “valuable consideration” of human organs, which also includes most human tissues. However, the Act also provides an important exception:

“The term ‘valuable consideration’ does not include the reasonable payments associated with the removal, transportation, implantation, processing, preservation, quality control, and storage of a human organ or the expenses of travel, housing, and lost wages incurred by the donor of a human organ in connection with the donation of the organ.”

### Federal Regulation

Human tissue intended for transplantation has been regulated by the FDA since the agency issued interim requirements in the Code of Federal Regulations (CFR) that became effective on December 14, 1993.<sup>78</sup> This initial regulation (21 CFR 1270) required certain infectious disease testing, donor screening, and record-keeping to prevent the transmission of HIV and the hepatitis viruses from human tissue used in transplantation.

On July 29, 1997, the FDA issued its final regulations (21 CFR 1270) that were broader in scope. The final rule required facilities that are engaged in recovery, screening, testing, processing, storage, or distribution of human tissues to ensure that specified minimum required medical screening and infectious disease testing have been performed and are documented.<sup>79</sup> The regulations also contain provisions for inspecting tissue facilities and for retaining, recalling, or destroying human tissue for which appropriate documentation is not available.

Also in 1997, the FDA announced a new, tiered, risk-based approach for regulating human cells, tissues, and cellular and tissue-based products (HCT/Ps).<sup>80</sup> This proposed regulatory scheme involved three new regulations that replaced the previous approach. The new regulations require establishment registration and product listing; donor eligibility requirements; and GTP.

The first of these, the “Establishment Registration and Listing for Manufacturers of Human Cellular and Tissue-Based Products” final rule, was published on January 19, 2001.<sup>81</sup> This rule requires that facilities participating in the recovery, screening, testing, processing, storage, or distribution of human tissue for transplantation must register with the FDA. These rules extend to all human tissues, imported and domestic, but hospitals or other clinical facilities that only receive and store human tissue for transplantation within their same facility are exempt.

The second set of regulations, “Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products,” was published on May 25, 2004, and became effective that day.<sup>82</sup> These regulations established screening and testing requirements relating to “cell and tissue donors for risk factors for, and clinical evidence of, relevant communicable disease agents and diseases.”

The culmination of the FDA’s new regulatory approach to tissue banking came on November 24, 2004, with the publication of its third and final rule, “Current Good Tissue Practice for Human Cell, Tissue, and Cellular and Tissue-Based Product



Establishments: Inspection and Enforcement.”<sup>83</sup> Similar to FDA’s cGMP regulations for pharmaceutical products<sup>84</sup> and medical devices,<sup>85</sup> the cGTP regulations require that every entity in the manufacturing process establish a quality program that addresses all the core cGTP requirements. These requirements are spelled out in 20 different sections.<sup>86</sup> In addition, the FDA has issued 10 guidance documents to supplement and interpret its regulatory requirements.

### State Laws and Regulations

Before 1968, no federal laws existed governing organ or tissue donation, and what state laws did exist varied considerably from state to state. In that year, to devise a system for donation of human tissue and organs, the National Conference of Commissioners on Uniform State Laws drafted and promulgated the Uniform Anatomical Gift Act (UAGA).

The UAGA is a model law for the states to adopt individually. The 1968 UAGA was enacted in all 50 states within 4 years. Almost 20 years later in 1987, a new version of the UAGA was drafted to address advances in transplant medicine and important issues that had developed, but it was enacted in only 26 states. A 2006 revision has been approved in approximately 30 states, as of June 2008.

The fundamental provisions of the UAGA govern the donor’s rights and the process for making a donation. It incorporates the NOTA prohibitions against buying and selling organs and deals with a multitude of other issues involved with donation.

In addition to following each state’s Anatomical Gift Act, tissue banks should also be aware of specific state laws or regulations. For example, the states of New York<sup>87</sup> and Florida<sup>88</sup> are the only two states that license and inspect tissue banks throughout the country. The oversight includes tissue procurement, donor screening and testing, processing, tracking practices, emergency procedures, labeling, facilities, and disposition of unused tissue. The states of California, Delaware, Georgia, Illinois, Maryland, and Oklahoma require tissue banks to register or be licensed by the state, or to have a permit. Of these, California and Maryland perform inspections to maintain licensure; however, they inspect only those tissue banks that are located within their state.

Many of these state laws and regulations are largely based on AATB *Standards for Tissue Banking*.<sup>89</sup> In fact, more than 20 states specifically reference or incorporate AATB accreditation or AATB *Standards* in their state laws or regulations. Six states (Illinois, Ohio, Oklahoma, South Carolina, Texas, and Virginia) and the District of Columbia require tissue banks operating in their jurisdictions to be AATB accredited.

### Industry Standards

What constitutes the appropriate standard of care is a question of law. Whether the defendant has met the standard of care is a question of fact that the jury determines. Over the years, trade associations and others have published standards or practice guidelines for their respective industries. These standards are developed to protect the public health and welfare and to serve

as a code of conduct for an organization’s members. Widely accepted, industry-created standards are evidence of the standard of care.

In tissue banking, apart from federal and state statutes and regulations, the principal source for determining the standard of care in tissue banking is the AATB’s *Standards for Tissue Banking*. These *Standards* are universally accepted and address virtually all aspects of tissue banking, including for example: institutional requirements; records; the consent process; donor eligibility; retrieval; processing; containers; storage; labeling; distribution; packaging; recalls; standard operating procedures; staff; facilities; equipment; quality assurance; quality control; testing; and release. In addition, the AATB has also published four guidance documents to supplement the *Standards*.

Because blood centers and transfusion services may store and provide tissue, the AABB *Standards for Blood Banks and Transfusion Services*<sup>90</sup> includes requirements for managing tissue. Those requirements are found primarily in the general quality standards, the storage and transportation standards, and record retention standards. Efforts have been made to harmonize AABB tissue standards with those of AATB. AABB also provides guidelines and a handbook on tissue management by hospitals, rather than collection facilities.

### Legal Issues

Legal issues relating to tissue donation and transplantation are very similar to those of blood banking and transfusion medicine. Both involve the transfusion or transplantation from a donor to a recipient. Both are regulated health-care industries where industry standards play a major role. Claims involving the negligent infliction of injury to a donor or a recipient are common to both settings, as are issues involving consent, violation of standards and accreditation, and even issues surrounding antitrust and patient privacy.

In addition, several states have enacted the tissue equivalent of blood shield statutes.<sup>91</sup> These laws deem tissue banking to be the provision of a “service” and not the sale of a “product.” As such, they provide statutory immunity from claims for “strict liability” or “breach of an implied warranty of merchantability or fitness for a particular purpose.”<sup>92</sup>

As medical science has uncovered more uses for donated human tissue and as new tissue-engineered products enter the health-care arena, the issue of ownership has surfaced.<sup>93</sup> As described above, in the case of *Moore v. Regents of University of California*,<sup>71</sup> the California Supreme Court determined that a person has no property interest in his or her cells after they have been removed from his or her body. The court noted that California laws bestow no property rights on patients for their tissue that has been surgically removed. In addition, the court found that the cell line was “both factually and legally distinct from the cells taken from (the plaintiff’s) body.” Perhaps more significantly, the court stated that:

“The extension of conversion law into this area will hinder research by restricting access to the necessary raw materials [and]

destroy the economic incentive to conduct important medical research.”

In *Greenberg v. Miami Children's Research Institute*,<sup>94</sup> the plaintiffs were families suffering from Canavan disease, a degenerative affliction of the central nervous system. They created a patient registry and engaged a physician to conduct research. The physician successfully identified the gene and developed tests for the disease. He also obtained a patent for the relevant genetic sequence for his institutional employer so that it controlled genetic testing for this disease. The plaintiffs sued, claiming that they had not provided resources for the economic benefit of the defendants.

Much like the California Supreme Court's ruling in *Moore*, the Florida trial court failed “to find a property interest in body tissue and genetic information voluntarily given to defendants.”<sup>94</sup> The judge also determined that there was no liability for conversion because the samples, used only for medical research, were distinct from the patented genetic sequence. Finally, the court also voiced a concern that finding for the plaintiff would “cripple medical research.”

A variation on a theme occurred in a recent decision involving a prostate cancer researcher who moved from Washington University in St. Louis to Northwestern University in Chicago and wanted to take his tissue samples with him. In *Washington University v. Catalona*,<sup>95</sup> the university sued to retain the samples. Testimony revealed that the researcher in question sent letters to the patients asking them to sign a second consent form releasing their samples to his new institution.

Based on the holdings in *Moore* and *Greenberg*, the court remarked that a person retains no rights in his or her tissue once it is removed from their body. It is a gift, and the issue was to whom these patients gave their tissue samples. The court decided that the gift of tissue was made to Washington University and not to the researcher—a finding that was upheld on appeal.

The rapid growth of tissue activities is bound to give rise to novel legal issues, with the technical requirements differing from those applicable in the blood banking and transfusion medicine arena. Nonetheless, case law relating to the legal requirements for defining when a duty arises and the applicable standard of care are likely to be influenced by those areas.

## Legal Considerations for HIPAA Privacy Rule Compliance

Blood/tissue collection and transfusion/transplantation services are subject to myriad regulatory schemes. Few produce as much confusion as the regulations governing patient privacy.

The Health Insurance Portability and Accountability Act of 1996 (HIPAA) authorized the DHHS to promulgate patient privacy standards.<sup>96</sup> In December 2000, the DHHS Secretary issued the first national privacy standards to protect individually identifiable health information (the Privacy Rule)<sup>97</sup> intended to accomplish three goals:

1. To protect and enhance the rights of consumers by providing them access to their health information and controlling the inappropriate use of that information.
2. To improve the quality of health care in the United States by restoring trust in the health-care system among consumers, health-care professionals, and the multitude of organizations and individuals committed to the delivery of care.
3. To improve the efficiency and effectiveness of health-care delivery by creating a national framework for health privacy protection that builds on efforts by states, health systems, and individual organizations and individuals.

The privacy rule creates a variety of affirmative obligations for covered entities and subjects covered entities to potential civil and criminal liability for violations.

Although this regulatory framework has broad subject matter coverage, Congress and the DHHS have excluded certain transactions from HIPAA and its regulatory progeny. Furthermore, when it adopted the final rule in December 2000, DHHS removed language that would have defined the procurement and banking of blood and tissue as a “health-care” activity.<sup>97</sup> This has resulted in many challenging legal issues unique to the field of blood/tissue collection and transfusion/transplantation services. Therefore, practitioners in this field must make individualized assessments of who they are and what precisely they do to determine HIPAA applicability and the scope of their obligations under HIPAA.

### Determining HIPAA Coverage

HIPAA applies only to individuals or organizations that conduct certain specified transactions. Organizations first must evaluate whether HIPAA applies before introducing or modifying their privacy policies. Because there are several exceptions to coverage, determining HIPAA applicability is complex.

### Who or What Is a “Covered Entity”?

Covered entities are divided into three groups: health plans, health-care clearinghouses, and health-care providers.<sup>98</sup> Each group has separate organizational obligations and restrictions under the privacy rule.<sup>99</sup> Coverage of health-care providers is the broadest category of coverage and applies equally to individuals and organizations. If an organization or person in the blood/tissue collection and transfusion/transplantation field qualifies as a covered entity, it will likely fall within this category.

To evaluate whether an individual or organization is a covered entity, one must determine: 1) whether he/she/it falls within the definition of health-care provider; 2) whether he/she/it transmits health information in electronic format; and 3) whether the information transmitted relates to a covered transaction.

The term “health-care provider” includes specific types of providers, specific types of services, and a catch-all that extends coverage to any person or organization who furnishes, bills, or makes payments for health care in the normal course of business.<sup>98</sup> A blood bank that does not provide patient-related services (such as therapeutic apheresis) would not normally fit

within the definition of a health-care provider. An organization that provides blood transfusions or tissue transplantation services or dialysis treatment or dialysis supplies and equipment, however, likely would meet the health-care provider definition.

A health-care provider is not covered by HIPAA unless the provider transmits personal health information (PHI) in electronic form or stores information electronically. An organization probably would still be covered even if it used only printed documents, because many third-party payers require the electronic submission of documents.

The last element to determine covered entity status requires PHI to be related to a covered transaction. Covered transactions applicable to blood banks include: health-care claims or equivalent encounter information; health-care payment and remittance advice; coordination of benefits; health-care claim status; eligibility for a health plan; health plan premium payouts; or referral certification and authorization.<sup>98</sup>

Although many organizations and practitioners in the collection and transfusion industry might seem to be covered entities, there are some exceptions built into the health-care provider definition.

### What Is the Procurement Exclusion?

As noted above, during the regulatory process, the DHHS regulations implemented a “procurement exclusion,” which applies directly to blood/tissue collection:

[T]he procurement or banking of organs, blood (including autologous blood) sperm, eyes, or any other tissue or human product is not considered to be health care under this rule and the organizations that perform such activities would not be considered health-care providers when conducting these functions.<sup>97</sup>

Because procurement is not intended for the health care of the donor, HIPAA is inapplicable. Therefore, if an organization is in the business of collection only, it is not considered a health-care provider and the HIPAA privacy rule does not apply.

Some commentators argue blood/tissue collection centers are always covered health-care providers. The view is premised on an overly broad reading of HIPAA that contends that coverage is extended automatically by virtue of handling PHI. This reasoning constructs an implied purpose that does not comport with the careful limitations on the HIPAA provisions as drafted.

Blood/tissue collection centers that perform patient-related support services, such as transfusions, diagnostic testing or therapeutic phlebotomy, in addition to procurement, may be covered entities as described below.

### How Does HIPAA Apply to Organizations with Mixed Transactions?

If an organization provides both covered services and noncovered services, such as procurement, it may choose either to comply with the HIPAA privacy rules all the time, or to self-designate as a “hybrid entity” and observe HIPAA regulations only when engaging in covered transactions.<sup>98</sup>

Some organizations and practitioners have decided to comply fully with HIPAA privacy regulations all the time regardless of HIPAA’s applicability to all their transactions. They choose this option, among other reasons, to prevent internal separation of functions or any administrative restraints on the free-flow of information internally.

Blood/tissue collection services should not be classified as covered entities unless they chose this classification because the broader organization performs covered transactions and the overall organization chooses to be HIPAA-compliant across the board. If an organization chooses to classify itself as a covered entity, the entity is subject in its entirety to the privacy rule obligations.

By contrast, a hybrid entity may limit its compliance to only covered transactions.<sup>97</sup> This method of compartmentalized compliance is known as the health care component approach. This approach grants flexibility to organizations that provide both covered and noncovered functions, by creating modified compliance obligations. For example, a blood bank that also provides therapeutic apheresis services may comply with HIPAA in providing just those services, while ignoring HIPAA for noncovered functions, such as procurement.

Overall, evaluating whether an organization meets the covered entity definitions requires a detailed, fact-specific analysis of its business functions. It also requires an organization to assess the particular benefits, if any, of operating as a hybrid entity. Because of the complexity of HIPAA compliance implementation for hybrid organizations, there will be unique legal concerns for each entity to consider. Therefore, it is prudent to consult counsel when assessing coverage and the scope of an organization’s HIPAA obligations.

### HIPAA Privacy Rule Protections

The privacy rule entitles health-care patients to certain rights and requires covered entities to protect the disclosure and use of individually identifiable health information.

### What Information Is Protected?

Individually identifiable health information maintained, created, transmitted, or received by a covered entity is protected by the privacy rule. Under HIPAA, the information is protected when: 1) the information is created or received by a covered entity; 2) the information relates to the past, present, or future physical or mental health of an individual; the provision of health care to an individual; or the past, present, or future payment for the provision of health care to an individual; and 3) the information individually identifies the recipient of care or contains sufficient information that a person reasonably believes can be used to identify the individual.<sup>98</sup>

The privacy rule is triggered only when an organization is a covered entity or a business associate of a covered entity. For example, a health assessment survey completed by a donor in the course of blood donation is not considered PHI, *if* the blood bank is not a covered entity or has not agreed to be a business associate of a covered entity.<sup>100</sup> Where the transaction is procurement only, the HIPAA privacy rule protections do not attach, unless the blood/tissue bank has opted to comply with HIPAA as described above.

In addition, the privacy regulations protect covered entities who de-identify or strip PHI of individually identifiable information. The privacy rule creates a safe harbor when a covered entity divulges PHI, if the individually identifiable information has been properly removed. The protection of this process, known as “de-identification,” can be obtained in one of two ways: 1) preliminarily obtaining the opinion of a qualified statistician that the risk of identification of an individual is small<sup>101</sup> or 2) removing the statutorily specified identifiers that could potentially give rise to identification.<sup>101</sup> Absent de-identification, PHI remains protected and may subject a covered entity to liability upon improper use or disclosure.

### What Protections Are Afforded PHI?

The privacy rule imposes a general prohibition on the use or disclosure of PHI by covered entities, except as expressly authorized or required by statute. The terms “use” and “disclosure” are separately defined. Use applies to protected health information when it is shared, applied, analyzed, examined, or utilized *within* a covered entity, whereas disclosure applies when the information is released, transferred, divulged, provided to, or accessed from *outside* the entity.<sup>98</sup> A covered entity should differentiate between the terms to evaluate its duties and limitations.

The privacy rule expressly establishes circumstances for permissible use and disclosure. Within these parameters, most decisions to use or disclose PHI are at the discretion of the covered entity. There are two exceptions, however, both of which impose an obligation to disclose: 1) upon request by the individual who is the subject of the protected health information, or 2) to the DHHS during an investigation for regulatory enforcement.<sup>102</sup>

Generally, the conditions on use and disclosure are restrictive and vary depending on the recipient, the purpose, and other attendant circumstances. Some uses and/or disclosures may be authorized absent an individual’s consent, some require the individual’s permission, while other instances require the covered entity to provide notice and opportunity for the individual to object. Instances where authorization is not required deal primarily with treatment, payment and health-care operations,<sup>103</sup> or statutorily designated public priority purposes.<sup>104</sup>

These provisions are intended to restore control of PHI to the patient by granting the individual the authority to authorize or request greater or lesser privacy protections. Any disclosure not mandated or permitted by the regulations requires individual authorization.<sup>105</sup> Therefore, a covered entity should always have a sample authorization at its disposal. The authorization must be written in plain language, identify the information to be used or disclosed, identify the entity or person disclosing and receiving the information, contain an expiration date or event, and describe how an individual may revoke authorization. However, a covered entity may implement more restrictive policies on use and disclosures if it wishes, such as requiring authorization for transactions where it would not otherwise be required. Similarly, a patient is entitled to request more comprehensive restrictions, even though a covered entity is not bound to agree.

The privacy rule also grants patients new rights not otherwise available under federal law. An individual now has the right to access his or her PHI. This creates an obligation for a covered entity to facilitate a right of inspection or a right to copy the health information.<sup>106</sup> A covered entity may deny that request in the statutorily proscribed manner and situations, but the covered entity is often required to provide reasons for that denial to the individual.<sup>106</sup> In addition, the individual now has a right to amend his or her PHI.<sup>107</sup> Also, the individual is entitled to receive notice of the covered entity’s privacy practices<sup>108</sup> and an accounting of disclosures upon request.<sup>109</sup> As a result, covered entities must implement a “privacy policy,” place their patients on notice, and maintain a history of disclosures.

### How Does a Covered Entity Implement Its Privacy Policy?

A comprehensive privacy policy addresses each of the situations where disclosure and use of PHI is possible and develops and implements adequate procedures to prevent noncompliance. The policies and procedures at a minimum should cover use and disclosure requirements for PHI the covered entity creates, maintains, receives, or transfers. The policy should also address procedures for analyzing and acting on requests for uses and disclosures, and for managing an individual’s invocation of his or her privacy rights. A covered entity has a continuing obligation to ensure compliance. By performing periodic assessments or audits of all information collected, maintained, and received by the organization, the entity can better minimize risk of liability.

A covered entity must provide notice of its privacy policy<sup>110</sup> and include therein certain additional administrative requirements. Thus, a covered entity must designate a “privacy official,” who is responsible for developing and implementing policies and procedures, receiving complaints, and providing individuals with notice of the privacy policy.<sup>110</sup> Also, the privacy official or a designated contact person must document all complaints and implement a procedure for receiving and addressing those complaints.<sup>110</sup> A covered entity must also ensure and keep a record that its workforce, including employees, volunteers, trainees, and interns, are appropriately trained on the policies and procedures governing PHI to carry out their respective functions.<sup>111</sup> A consultant or law firm can facilitate the training for the entity, or the covered entity may choose to conduct the training in-house.

Additionally, a covered entity’s privacy policy must take into consideration state law. Because HIPAA authorizes states to enact legislation that provides for greater protections of the use or disclosure of PHI and perhaps more expansive individual rights, an organization should always consult with counsel to determine what additional requirements or restrictions, if any, are applicable.

### HIPAA PHI Safeguards

Reasonable safeguards are necessary to ensure that PHI is protected from intentional or unintentional disclosure.<sup>112</sup> Although the privacy rule does not require a covered entity to ensure the elimination of *all* risks of incidental use or disclosure, a covered

entity is expected to develop and implement minimum necessary policies and risk management strategies.

### What Is the Minimum Necessary Standard?

The minimum necessary standard, under the privacy rule, limits the amount of PHI that may be used, disclosed, or requested.<sup>113</sup> The covered entity must make reasonable efforts to limit the information to the minimum amount necessary to accomplish the intended purpose of the use or disclosure.<sup>113</sup> A covered entity is not required to perform individualized assessments of each use and disclosure. Instead, the covered entity must implement plans and policies that ensure the minimum necessary standard will be met. For example, covered entities should implement access policies on a “need to know” basis for their personnel by identifying persons or classes of persons within the organization and limiting their access to PHI to only the information necessary in the performance of their job duties.<sup>114</sup> However, the minimum necessary standard excludes disclosures intended for the purposes of treatment.

### How to Implement a Risk Management Plan

HIPAA provisions also require covered entities to implement a risk management plan. An appropriate risk management plan should implement a variety of administrative, technical, and physical measures to protect the use or disclosure of PHI in contravention of the privacy policy.<sup>112</sup> Traditional measures such as speaking quietly with a patient, avoiding the use of names, and isolating or locking file cabinets and record rooms are common practices. In addition, HIPAA requires covered entities to implement electronic security measures, such as password protection, limiting access, both on-site and remotely, enacting provisions governing portable electronic storage devices (laptops, CDs, and external hard drives), and installing fire walls and virus protection software.<sup>115</sup> A comprehensive risk management plan should address all facets of information flow, including access, storage, and transmission of PHI.

A covered entity should consider including mitigation provisions in the privacy policy. Should the disclosure of PHI occur, unintentionally or intentionally, the privacy rule requires a covered entity to mitigate, to the extent practicable, any harmful effect that is known of a use or disclosure of PHI in violation of its policies.<sup>116</sup> A covered entity should also consider implementing deterrence measures, such as sanctions against its workforce, for those who fail to comply with the privacy policies and procedures.<sup>117</sup>

### What Additional Safeguards Do Hybrid Entities Require?

Hybrid entities have further obligations to ensure security of PHI within the entity. HIPAA prohibits the disclosure of PHI by the health-care component to a noncovered component of the covered entity, in accordance with the privacy rule.<sup>118</sup> Moreover, a covered entity’s employees or persons within its workforce must not disclose PHI created or received in the course of work for the health-care component during their employment in a different

component.<sup>118</sup> Accordingly, the covered health-care component should develop appropriate privacy policies and procedures to minimize risk of internal disclosure.

### Business Associate Agreements

Even if a blood collection service does not meet the definitional requirements of a covered entity, HIPAA may still apply based on requirements relating business associate agreements.

### What Are Business Associates and Business Associate Agreements?

A business associate is not a member of the covered entity’s workforce, but performs or assists a covered entity in the performance of a function or activity involving the use or disclosure of PHI.<sup>98</sup> For instance, covered entities, such as hospitals, may require that a collection service enter into a business associate agreement before entering into contractual arrangements for the supply of blood and tissue or related services. Whether such an agreement is necessary turns on an examination of what the collection service is asked to do for the covered entity.

A business associate agreement is required only when an organization engages in activities involving the use or disclosure of individually identifiable health information. Those activities include, but are not limited to, claims processing or administration; data analysis, processing, or administration; utilization review; quality assurance; billing; benefit management; practice management, and repricing.<sup>98</sup> Because the procurement exclusion expressly exempts collections of blood and tissue from HIPAA, supply alone should not result in the exchange of protected health information. To accurately evaluate whether a business associate agreement is required, the parties should examine the extent of the services provided and the content of the information exchanged.

If an organization in the field of blood or tissue procurement and transfusion services designates itself as a covered entity, the organization has the added obligation to require business associate agreements. Business associate agreements govern the use or disclosure of PHI and are designed to ensure that business associates use PHI only for the purposes that they are engaged to perform or as otherwise required by law. Business associates must safeguard the information from impermissible uses and disclosures, report any such disclosures to the covered entity, and otherwise assist the covered entity in complying with HIPAA requirements.

### Are Third Party Business Services Covered?

A business entity may also provide business-related services, such as legal, actuarial, accounting, consulting, data aggregation, management, administrative, or financial services to or for a covered entity that do not involve health care but may involve access to PHI.<sup>98</sup> A covered entity should negotiate a business associate agreement with any third parties that provide these types of services, such as a certified public accounting firm that provides accounting or auditing services or an attorney whose legal services involve access or review of PHI.

In addition, HIPAA specifically includes accreditation as a covered service that gives rise to a business associate relationship. Therefore, covered entities or health-care components must ensure that PHI shared during the accreditation process is similarly protected from unlawful use or disclosure through a business associate agreement between the covered entity and the accreditation authority.

A covered entity has a continuing obligation to ensure its business associates comply with the terms of the agreement. A covered entity may be liable for actual knowledge of noncompliance. When instances of noncompliance do arise, the covered entity should consider appropriate actions to mitigate noncompliance or even consider terminating the relationship. In some instances, the covered entity must report the noncompliance to the DHHS.

### Privacy Implications for Research

Business associate agreements are not necessary for researchers. HIPAA creates an entirely different set of requirements for researchers and covered entities who elect to use PHI in their research. Traditionally, research involving human subjects has been governed by the DHHS Protection of Human Subjects Regulations<sup>119</sup> and/or the FDA human subject protection regulations, depending upon the source of funding for the research.<sup>120</sup> Now, HIPAA contains additional provisions for researchers.

### What Research Is Covered by HIPAA?

HIPAA extends the protections of the privacy rule to research groups that are covered entities and to research conducted by covered entities. Certain types of research, such as quality improvement research, may fall within the category of conducting patient care and subject the research to HIPAA in its entirety. In contrast, research performed that does not include the use of PHI may not meet HIPAA's threshold standard for coverage. As a result, organizations should preliminarily determine whether they will be subjected to HIPAA's privacy regulations before authorizing or conducting research.

### What Research Use or Disclosure Is Permitted

#### Absent Consent?

HIPAA establishes guidelines for research use and/or disclosure of PHI without authorization or consent. A covered entity is permitted to disclose PHI to a researcher solely to prepare a research protocol or for research involving only PHI of decedents.<sup>121</sup> All other PHI uses and/or disclosures require: 1) approval of alteration or waiver by an IRB or privacy board, 2) a limited data set agreement, or 3) individual authorization.

#### Privacy Board

A privacy board is an additional review body formed with the limited authority to evaluate requests for waivers of PHI. Board members should possess varying backgrounds of professional competency to review the effect of the research protocol on the

individual's privacy interests.<sup>121</sup> No member participating on the board can have a conflict of interest, and at least one member must not have affiliations with the covered entity or the entity sponsoring or conducting the research or its affiliates.<sup>121</sup>

A covered entity may use or disclose PHI only when it obtains a valid, documented waiver from the IRB or privacy board. The waiver should identify the board, the date of approval, the PHI needed, and a statement of review and approval procedures.<sup>122</sup> The researcher requesting the waiver must show that the research practically could not be conducted without the waiver or approved alteration.<sup>122</sup> In addition, the researcher must show that the use or disclosure will not involve more than a minimal risk to the individual's privacy. The burden to protect individual privacy requires the researcher to implement a risk management plan to protect PHI from improper use and disclosure, to destroy PHI at the earliest opportunity, and to provide adequate written assurance that PHI will not be reused or disclosed to any other person or entity.

### Limited Data Set Agreement

A researcher may also enter into a limited data set agreement with a covered entity. This allows the covered entity to disclose a limited set of PHI without individual authorization. Although the PHI is not de-identified, portions must be removed, such as names, street address, social security numbers, and account numbers.<sup>123</sup> Additionally, the research agreement must contain provisions that limit the use of the data set for particular purposes given, establish who is permitted to use and receive the data, ensure the security of the data, report any unauthorized uses or disclosures, ensure that agents and subcontractors agree to the same restrictions and conditions that apply to the recipient and not identify the information or use it to contact any individual.<sup>123</sup>

### Individual Authorization

A researcher may also use or disclose PHI when a research participant provides individual authorization. The researcher must first ensure, however, that the authorization meets specific HIPAA provisions governing authorizations.

HIPAA authorizations should not be confused with the separate legal requirement of informed consent. A researcher may decide to use one single form, known as a compound authorization, that contains both the provisions for consent and the authorization pursuant to HIPAA, or two separate documents, a "stand-alone" HIPAA authorization and a consent document. If a researcher decides to use a compound authorization, this document must undergo IRB review, given the element of consent. Otherwise, a HIPAA stand-alone authorization does not require review.

Additionally, a covered entity must determine when an authorization is required. For instance, when a tissue sample is used for clinical research *outside* of the tissue repository, HIPAA documentation must be obtained. It is not as clear, however, whether the de-identification of this tissue sample avoids HIPAA coverage.

Because HIPAA provisions authorize a covered entity to identify specimens according to a code format, de-identification of the sample should be possible. As long as the code is not related

to information about the donor, arguably tissue and/or blood samples may be unlinked from donor information. In these situations, researchers are struggling with the applicability of informed consent. Is consent necessary in this situation? Is the donor's privacy adequately protected by the safeguards in place? Does the de-identification safeguard use or disclosure in accordance with HIPAA? Will one-time consents be honored? Legal challenges certainly exist and the regulations governing research of tissue and blood will continue to be shaped in the upcoming years.

## Donor Privacy Protections Under State Law

In addition, blood donor confidentiality is generally mandated by *state* law, regardless of HIPAA's applicability to a specific case. Several states have statutes or regulations expressly requiring the safeguarding of donor confidentiality.<sup>124</sup> In some instances, courts have found such a right to be footed in the state constitution.<sup>125</sup> Other courts have identified a privacy right based on public policy.<sup>126</sup> Because the specifics of these privacy protections vary from state to state, it is important to be acquainted with the relevant state law in a particular case.

One effect of these provisions has been to restrict the discovery that can be taken from blood donors in transfusion litigation. In one California case, the court prohibited the plaintiffs from conducting even "veiled" depositions of blood donors, who would have remained unidentified behind a screen, because the court felt that this would have violated the strict state law on donor confidentiality.<sup>127</sup> More commonly, courts will permit an anonymous deposition or a deposition upon written questions of a blood donor to be taken where the donor has been identified as a source of an infectious agent and the deposition is not simply a "fishing expedition."<sup>128</sup>

## HIPAA Summary

Understanding HIPAA imposes difficult challenges on blood/tissue banks and transfusion/transplantation centers, or any organization or person procuring, supplying, or studying human cell, tissue, and cellular and tissue-based products. Procurement activities alone should not be enough to trigger HIPAA coverage. Hybrid entities are complicated to manage, however, and collection centers may be required to enter into business associate agreements with hospitals and other covered entities. All practitioners in this area should seek advice and guidance when facing uncertainties in HIPAA compliance.

## Disclaimer

The authors have disclosed no conflicts of interest.

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6. See, eg, Kansas Stat. § 65-3701; New Hampshire Rev. Stat. § 507:8-b; New York McKinney's Public Health Law § 580.
7. See, eg, Ariz. Rev. Stat. § 12-561; Fla. Stat. Ann. § 766.202; Indiana Code § 34-18-2-14.
8. See, eg, *Wilson v. American Red Cross*, 600 So.2d 216 (Ala. 1992); *Coe v. Superior Court*, 220 Cal.App.3d 48 (1990); *Smith v. Paslode Corp.*, 7 F.3d 116 (8th Cir. 1993); *Smith v. American Red Cross*, 886 F. Supp. 1494 (E.D. Mo. 1995); *Estate of Doe v. Vanderbilt University, Inc.*, 824 F. Supp. 746 (M.D. Tenn. 1993). However, courts in the following cases found that blood centers were not "health care providers" for purposes of health care liability reform legislation: *Blood Bank of Delaware v. Price*, 748 A.2d 406 (Del. 2000); *Miles Lab v. Doe*, 556 A.2d 1107 (Md. 1989); *Doe v. American National Red Cross*, 500 N.W.2d 264 (Wis. 1993).
9. See, eg, Massachusetts Gen. Law Ann. 231 § 85K; Texas Civil Practice & Remedies Code § 84.006.
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With the emergence of AIDS during the early 1980s, the interest of the public as well as regulatory authorities in the safety of the blood supply increased dramatically and has remained a top priority. The vast majority of blood and blood components for transfusion in the United States are supplied by free-standing blood centers that collect blood from donors or process blood and blood components for transfusion. Some blood is collected in hospital-based blood banks located in a hospital laboratory with a transfusion service. The current good manufacturing practice (cGMP) concepts apply equally to both blood establishment types. The high level of regulatory oversight has been due, in part, to the public's low tolerance for risk, especially risk associated with blood transfusions. It also has been difficult for the public to accept that resources are limited and that reducing the risks of transfusions further will require trade-offs for risk reduction in other parts of the health-care system.<sup>1</sup> Finally, because biologic systems are imperfect, reduction of transfusion risks to zero is not possible, regardless of the resources dedicated to achieve such a goal. In addition, despite increasingly effective safeguards in process control, human error can still defeat even the most stringent control devices.

Compliance with the provisions of Title 21 of the Code of Federal Regulations (CFR) specifying cGMP requirements that outline production process control for the pharmaceutical industry continues to be the focus of inspections and guidelines by the Food and Drug Administration (FDA). Although guidelines do not exert the force of regulations, they provide establishments with the FDA's current thinking regarding the systems in blood centers to control production processes. Further, compliance minimizes the probability that ineligible donors will be accepted or that unsuitable blood components will be made available for transfusion. This is an even more restrictive concept than that used by the FDA to define reportable errors in which quality is

defined as "... never releasing an unsuitable unit for transfusion or further manufacture."<sup>2</sup>

Current good manufacturing practice principles embrace industrial rather than medical concepts. The subspecialty of transfusion medicine has been carved from other medical disciplines over the last three decades.<sup>3</sup> Because blood center operations traditionally have been viewed as medical rather than industrial enterprises, the need to implement cGMP in blood centers was not immediately obvious and the benefits were only slowly recognized. Further, implementation of cGMP has been resisted by some blood banking professionals because they believed implementation would be detrimental to transfusion medicine as a medical discipline.<sup>4,5</sup> Finally, it has been observed that health-care organizations are not ideally suited for modern industrial management.<sup>6</sup> Thus, blood centers have been required to separate operationally medical functions, such as providing consultations and patient care, from manufacturing activities, such as the manufacturing of blood components. The prevailing standards of medical care largely define the practice of the former, whereas cGMP regulations govern the latter.

This chapter opens with the history and the elements of cGMP and an introduction to blood center supplementation of cGMP with statistical process controls and process innovation. A synopsis of additional regulatory criteria to be met by facilities in the United States selling plasma for fractionation is described (as reflected by the guidance documents issued by the Pharmaceutical Inspection Convention/Pharmaceutical Inspection Cooperation Scheme known as PIC/S). In addition, the application of recent scientific advances such as molecular approaches will be addressed.

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**Historical Perspective**

The development and implementation of laws and regulations regarding drugs, biologic products, and medical devices are exceedingly complex. In the simplest terms, Congress passes broad enabling legislation and operational regulations are then

promulgated by regulatory agencies, primarily the FDA and Centers for Medicare and Medicaid Services (CMS) in the case of blood centers.

## Beginnings

Biologic products initially were regulated when Congress passed the Biologics Control Act in 1902.<sup>7,8</sup> Many provisions of this act established practices that remain part of current FDA law, more than 100 years later. Among these provisions is the requirement that a biologic product obtain premarket approval (ie, a license) for the article to be shipped in interstate commerce. Historically, all food and drug manufacturers were required to register their firms and products with the FDA. In order to ship biologic products in interstate commerce, however, both establishment and product licenses were required. A product license would not be issued in the absence of an establishment license nor an establishment license granted unless the firm manufactured at least one licensed product. In that scheme, a *responsible head* (under current terminology, an authorized official, see below) served as the contact between the firm and the FDA.

## Licensing Products and Establishments

Current regulations require that a firm file only a biologics license application (BLA) to ship blood products in interstate commerce.<sup>9,10</sup> In addition, the term *responsible head* was replaced by *authorized official*, and the responsibility for regulatory compliance rests with this person or persons designated to represent the firm. Firms that already held approved establishment licenses and product licenses were issued a new biologics license when the FDA received the first supplement to a firm's existing approved license. The approved product license along with the portion of the approved establishment license relevant to the requirements of the new regulation are deemed to constitute a biologics license under section 351 of the US Public Health Service (PHS) Act.<sup>11</sup> Although "user fees" do not apply to blood components at this time, the FDA has committed to work with sponsors to speed the process of biologics license regulatory review<sup>12</sup> and target timelines are applied to the review process despite the lack of a mandate. Guidance for industry about submissions is available from the FDA.<sup>13,14</sup>

The form used to apply for a license to market a new drug or biologic or to supplement an existing biologics license [Form FDA 356(h)], contains a certification section in which the applicant agrees, among other things, to comply with the cGMP provisions of the CFR. This certification clearly reinforces the emphasis by the FDA on cGMP.

The FDA has provided many guidance documents to ensure compliance with the requirements for BLA submissions.<sup>15</sup> This guidance partially echoes the scheme for interacting with the FDA about license changes as described in 21 CFR 601.12, which establishes reporting requirements.<sup>16</sup> The extent of FDA notification about a change is now categorized according to the significance of the change. Major changes, defined as presenting "... a substantial potential to have an adverse effect on the safety or

effectiveness of the product ..." require a supplement submission and approval (Prior-Approval Supplement/PAS) before distribution of a blood product in interstate commerce. Examples of changes (provided by the FDA) requiring this most stringent level of regulation include implementation of a new production process for a blood component or a change in contractor to perform a portion of the manufacturing process, including infectious disease testing of blood donors.

Moderate changes that present a "... moderate potential to have an adverse effect on the safety or effectiveness of the product ..." require notification of the agency (Changes Being Effected/CBE-30) at least 30 days before the distribution of a product affected by the changes. Included in this moderate category are changes such as implementation of a computer-assisted donor health history process or a change in manufacturer of automated donor apheresis equipment.

Moderate changes requiring supplemental submissions before distribution of a product affected by the change can also be made without waiting the 30 days (CBE). Labeling the application Supplemental Changes Being Effected (CBE) at the time of submission is required for this regulatory treatment. Examples of these changes provided by the FDA include the use of an FDA-approved standard operating procedure (SOP) of another licensed facility.

Minor changes are placed in the least stringent regulatory category. Such changes need only be included in the annual report. Examples of minor changes as outlined by the FDA might include modification of quality control procedures that are consistent with the manufacturer's instructions, changes in radiation equipment used by the applicant or a contractor, selection of a different leukocyte reduction filter or minor facility renovations.

From a practical standpoint, the changes brought by the single-license BLA have little impact on the day-to-day regulatory environment within which blood centers and transfusion services operate. The legal obligations with regard to cGMP remain unchanged, and the applicability of the cGMP elements described in greater detail below continues to expand. The FDA has encouraged licensees to communicate uncertainties about changes, including seeking guidance before implementing them.<sup>16</sup> It also encourages cover letters with submissions to improve communications between the agency and a blood establishment. As noted, the "authorized official" has assumed the previous vital role of the responsible head in such interactions, but the new terminology permits more flexibility in communication with the FDA because alternate authorized officials are acceptable.

## Recalls and FDA Enforcement Activities

Recall authority of the FDA essentially remains unchanged by the licensing process modifications (21 CFR 7.40-7.59) and applies equally to drugs, biologics, and medical devices. All products manufactured in violation of FDA requirements are subject to recall. Following revocation or suspension of a license, the FDA may also order a recall of products already distributed by the firm. In 1902 and 1903, the PHS, then the enforcer of the act, focused

**Table 64-1.** Examples of Early Regulatory Requirements\*

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- Immediate reporting of establishment changes.
  - Demonstration of personnel training and competency.
  - Maintenance of production records and controls.
  - Lot release of articles.
  - Labeling requirements.
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\* Used with permission from Timm.<sup>7</sup>

on suspension and revocation of licenses as the basic enforcement mechanisms for biological products, with unannounced inspections occurring at least every 2 years. This compliance approach continues to dominate current FDA enforcement philosophy in dealing with biological firms it views as noncompliant.

### Safety Initiatives

In 1905 Upton Sinclair published *The Jungle*, which described horribly unsafe and filthy practices in Chicago slaughterhouses.<sup>17</sup> The book is widely regarded as providing part of the impetus for the passage of the Pure Food and Drug Act of 1906. That law addressed the purity of food and drugs but neither their safety nor efficacy. In 1909, at a time when inspections of biologic establishments were conducted by the Marine Hospital Service, the concept of responsible head was developed. This person acted as a single point of interaction between the FDA and an establishment.

Between 1909 and 1919 additional regulatory requirements were formulated to supplement the Biologics Control Act of 1902 (Table 64-1). Many of these provisions, such as training, production records, control records, and lot release requirements, remain part of the regulations today. These provisions are key elements of cGMP.

In 1938, over 100 people died from a pharmaceutical company's elixir containing a toxic alcohol. Simple toxicity tests would have demonstrated that the formulation was unsafe, but such tests were neither required nor performed. The Federal Food, Drug, and Cosmetic (FD&C) Act passed shortly thereafter required that the sponsor of a product subject to licensure or new drug approval prove its safety as well as its purity.

In response to the severe congenital defects caused by thalidomide in the late 1950s, the FD&C Act was amended significantly in 1962 (Kefauver-Harris amendments). For the first time drugs were also required to be proven effective, and this proof could be obtained only by clinical trials approved by the FDA before their initiation. Unapproved drugs could not be distributed and given to patients until an investigational new drug (IND) exemption for the candidate drug was reviewed and accepted by the agency. These amendments completed the well-known approval or licensure triad: safety, purity, and efficacy.

### Short Supply—A Unique Form of Oversight

An unusual provision of the Code of Federal Regulations provides an additional pathway to ensure compliance with the regulations but at the same time relieves the FDA of many burdensome inspections. Title 21 CFR Section 601.22 permits

unlicensed biologic products, such as Recovered Plasma, to be shipped for further manufacture while transferring the inspection responsibility from the FDA to the buyer of products declared to be in short supply. This provision applies particularly to unlicensed facilities because it permits any registered establishment to ship unlicensed Recovered Plasma (separated from Whole Blood), but not Source Plasma (obtained by apheresis), in interstate or international commerce under Section 601.22, provided the buyer has documented that the product is in compliance with the provisions of the CFR and the source material specifications for the final, FDA-approved product.

In applying the requirements of cGMP to a firm, the distinction between licensure and registration is more technical than real. Licensure is required to ship products subject to license in interstate commerce or internationally. Registration is required for all blood and tissue establishments, including those located in hospitals, that manufacture (collect, store, process, or distribute) blood components for transfusion or further manufacture. Registration requirements for blood are outlined in 21 CFR, Part 607; license requirements are in 21 CFR, Part 601. Tissue for transplantation (including progenitor cells) requirements are found in 21 CFR Parts 1270 and 1271.

### Enforcement Options

A major regulatory action available to the FDA for enforcement includes injunctions and consent decrees. In the early 1990s, regulatory letters sent to some blood centers cited failure to comply with provisions of Title 21 CFR Section 211. Through these letters the FDA gave all the blood centers notice that they would be required to comply, not only with the cGMP portions of the Title 21 CFR 600 series (although technically parts, they are often referred to as series), but also with the provisions of the 200 series. This application of the more strict regulations by the agency was reinforced in many Form FDA 483s (the reports of observations by individual investigators left with blood centers following an FDA inspection). Frequently these observations suggested that the investigator(s) did not believe the establishment was in compliance with regulations of the agency, including cGMP.

A pattern emerged in which blood centers gradually were being regulated as pharmaceutical manufacturers. Section 210.2(a) clearly states that the CFR 600 and 200 series supplement one another. Only when there is a conflict does one take precedence, and in that case the more specific applies.

In 1991, the FDA published a memorandum that outlined the more common deficiencies of blood establishments.<sup>18</sup> Deficiencies were most frequently found in the areas of donor deferral, viral testing, SOPs, and computer systems operations. Through this memorandum, the agency gave blood centers additional notice of its regulatory intent; this intent continues to be reinforced by current actions of the agency.

In 1991, the FDA also published the first draft of proposed "Quality Assurance Guidelines for Blood Establishments" and held a workshop to receive input from the regulated establishments.<sup>19</sup> It became very clear at this workshop that cGMP

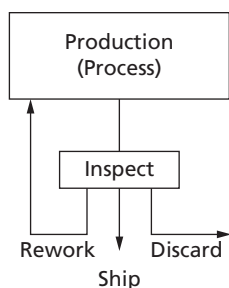


**Figure 64-1.** The detection model of quality control. Note the negative consequences of failed inspections.

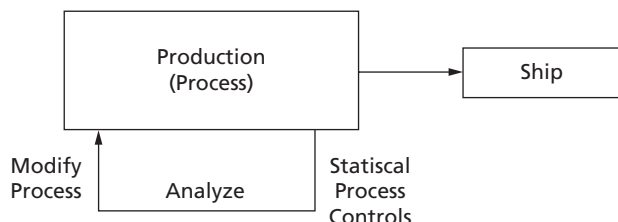


**Figure 64-2.** The prevention model of quality control. Note the similarity of this process with that of error management as diagrammed in Fig 64-4.

included quality systems and formed the cornerstone for FDA's intensified regulatory approach. It was reemphasized that blood centers producing blood and blood components are pharmaceutical manufacturers and would be regulated as such.

## Current Good Manufacturing Practice Rationale

As noted, cGMP is an industrial concept and, as such, its principles are rooted in industrial quality models.<sup>20</sup> In the traditional detection model for quality (Fig 64-1), testing of final product is used to discover production defects and weed out unsuitable products. Products are shipped with the knowledge that some percentage has undetected defects. It is left to the purchaser to discover a defective product and seek replacement. Detection has been the quality model generally used by health-care facilities.<sup>21</sup> But Deming, as well as others, emphasized that testing final products cannot improve manufacturing quality—it can only describe it.<sup>22</sup> The basis for this belief lies in the calculus of errors. Even a small variance in each process can result in major product variation if numerous production steps are required. Thus, the detection model is no longer viewed as ideal for controlling biomedical manufacturing establishments. Product testing as the primary means of controlling quality in blood centers is particularly unsuitable because the quality of the raw materials—blood given by volunteer donors—cannot be completely controlled. Furthermore, because each donation becomes a “lot” in the traditional pharmaceutical production sense, it is not feasible to perform additional tests on all products manufactured from each donation, that is, each lot. Finally, a blood product defect, such as contamination by an infectious agent that could have been intercepted by laboratory testing,

**Table 64-2.** Elements of Current Good Manufacturing Practice

- 
- Standard operating procedures.
  - Record-keeping.
  - Personnel management.
  - Calibration.
  - Validation.
  - Labeling.
  - Error management.
  - Quality control and auditing.
  - Facilities and equipment.
  - Process and production change control.
- 

may be discovered in a transfusion recipient only after disastrous consequences have ensued many months later.

In contrast to the detection model, the prevention model (Fig 64-2) seeks to obviate manufacturing variation by rigidly controlling manufacturing processes. In this model, it is assumed that employees will have lapses but that the design of the manufacturing systems will prevent such lapses from resulting in the release of components unsuitable for transfusion. This model is particularly apropos for blood centers because, as noted, the quality of the raw materials cannot be predicted or completely controlled; likewise, each lot (a component) cannot be exhaustively tested. However, the manufacturing processes can be controlled with the objective of reducing lot-to-lot variation. The cGMP concepts provide a roadmap for production controls and, as such, have been embraced by blood centers. The power of the elements of cGMP rests in their simplicity and universality. As described below, each element can be applied to any manufacturing function, or skill box. In the context of this chapter, skill box refers to the smallest discrete step in a production process that can be described, characterized by the FDA, and controlled. The elements of cGMP are listed in Table 64-2. This division into elements is arbitrary but is based on an analysis of commonality of the requirements of Title 21 CFR as they apply to all aspects of blood center operations. Thus, it is irrelevant whether they are applied to blood collection or to infectious disease testing; each element is applicable for each discrete blood center operation. The principle driving the elements rests in flow charts of operational production processes. In Fig 64-3 the general flow diagram of the production of platelets is shown. Optimally, cGMP elements are applied to each skill box in the manufacturing process. However, the cGMP elements should be viewed in the context of a blood center's entire production system. The safety of the blood supply is protected only by their application to the total system. The levels of protection (Table 64-3) of the blood supply create a safety system with intentionally designed redundancies.

### Standard Operating Procedures [§§211.100(a) and 211.22; §606.100(b)]

Management of standard operating procedures lies at the heart of quality manufacturing and cGMP compliance. For the purposes of this chapter, quality is narrowly defined as never

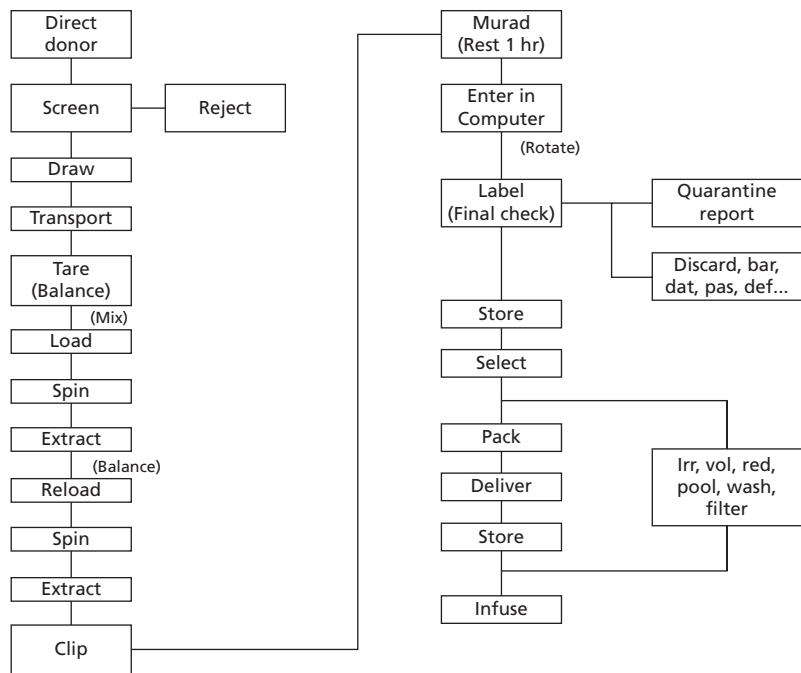


**Figure 64-3.** Simplified flow chart of platelet production. (Used with permission from Zuck.<sup>23</sup>)

**Table 64-3.** Levels of Protection of the Blood Supply

- Donor self-exclusion.
- Screening questions.
- Viral and surrogate testing.
- Deferral registries and duplicate search.
- Unit exclusion strategies.
- Confidential unit exclusion.
- Call-back programs.

releasing a product unsuitable for transfusion or further manufacture. Because blood and plasma centers are required to follow their own SOPs, each SOP creates unique regulatory requirements for that center. From a regulatory standpoint, this concept is significant in that compliance standards and regulatory requirements are created by individual center SOPs. Procedures for documentation control are essential to ensure that all SOPs, including any changes, are reviewed and approved by the quality unit. Any deviation from established procedures should be documented and investigated. Such investigation should determine whether the deviation had an impact on quality. The results of such investigation should be reviewed and approved by the quality unit. A list of responsibilities of the quality unit is found in Table 64-4.

Current good manufacturing practice describes both the production methods used by blood centers to manufacture components and the manufacturing process controls in place. Section 211.22 requires approval of all SOPs by the quality control unit. As defined by the FDA, the quality control unit is an

**Table 64-4.** Quality Unit Responsibilities<sup>24</sup>

**Attributes of the Required Quality Unit**

- Responsibilities are described in writing.
- Is independent of production.
- Is involved in all quality-related matters.
- Reviews and approves all quality-related issues.
- Has adequate analytic control facilities at its disposal.

**Quality Unit Responsibilities Should Include, But Not Be Limited to, the Following**

- Approve specifications.
- Approve test procedures, including process controls.
- Approve validation plans, protocols, or equipment.
- Review changes in product, process, or equipment and determine if revalidation is required.
- Approve specification changes, sampling plans, and test procedures.
- Approve sampling procedures.
- Approve reference standards.
- Conduct analytic investigations and evaluate results.
- Approve testing materials.
- Provide analytical reports.
- Approve or reject intermediates and active pharmaceutical ingredients manufactured, processed, packed, or held under contract by another establishment.
- Gather data to support retest dates (stability testing).
- Evaluate and approve contractors.
- Review batch records.
- Review complaints.
- Dispose of materials not meeting specifications.
- Dispose of materials returned to the establishment.
- Perform internal and external audits.
- Perform periodic assessments of procedures, policies, and responsibilities within the establishment's manufacturing and control operations.

organizational entity with defined responsibilities and authority for controlling, through checking or testing, that specifications are met and quality systems are maintained. The FDA has explained that the quality control unit cited in 21 CFR 211 is equivalent to what is now called a quality program, quality system, or quality assurance department. However, the principle of having a single “head” responsible for quality issues remains extant, without regard to what designations appear on an organizational chart. All matters that relate to quality should funnel to a single person or group with broad knowledge of the entire blood center operation and access to the information necessary to assess the impact of proposed changes. These principles emphasize the importance with which the FDA views both the quality unit and SOPs.

Consensus has not developed about the level of detail that should be contained in blood center SOPs. It is generally agreed that they should be sufficiently explicit that a qualified technical person could, with minimal training, perform the described procedure and manufacture products that meet all requirements. Highly detailed SOPs require more frequent revision and are much more likely to be violated by center personnel, a failing that makes blood centers vulnerable during inspections, particularly when inspectors actually observe performance of procedures.

Every provision of a center’s SOPs must reflect the manufacturers’ instructions for licensed or approved systems used in the center. It is strongly recommended that planned deviations from a manufacturer’s instructions occur only after submission to the FDA of the proposed alternative; however, it should be noted that FDA does not generally look favorably on such changes as evidenced by the requirement at 21 CFR 606.65(e) for following the manufacturer’s directions for use. Difficulties associated with following a manufacturer’s directions for use should first be addressed with the manufacturer. Further, 21 CFR 211.100 requires that all deviations from SOPs, whether planned or inadvertent, must be documented. Change control of SOPs remains one of the more difficult issues for blood center management. Standard operating procedures to manage procedural changes are critical when implementing new and revised processes. Both meticulous change control and periodic review of SOPs during audits are essential to prevent “drift” in manufacturing processes.

A reference resource manual of SOPs of member centers maintained by America’s Blood Centers has proven to be a useful resource for supplying benchmarks for blood centers.

### **Record-Keeping (§§606.160, 606.170, and 211.180 through 211.198)**

Of the cGMP elements, record-keeping is perhaps one of the most important, particularly from the perspective of preparing for inspections or defending a center’s actions if legal issues arise. During an inspection, it is assumed that an activity not documented was not performed. In general, the record-keeping requirements of the 21 CFR 200 series are more detailed and rigorous than those of the 600 series. However, the provisions of both series require concurrent, legible, indelible documentation of each production step (linked to the operator by

dated signature or initials) and an audit trail for any changes subsequently made to records. These requirements apply to both paper records and electronic records but additional requirements for electronic records exist and are discussed below. Records for every lot must permit traceability and trackability of a product by both the manufacturer and the FDA during inspections and recalls. The latter may require reconstruction of manufacturing (including labeling) of a product that has been linked to an adverse reaction or other problem. Both the 21 CFR 200 and 600 series require that records of adverse reactions, deviations, and complaints be maintained. These records may be scrutinized during inspections because they may indicate weaknesses in the manufacturing controls of an establishment. It is essential that the records of these events include an evaluation of the problem to determine if a root cause analysis is warranted. If an investigation is performed, maintaining a detailed description of the process is critical. The records should document who made the decisions concerning whether the events also required a deviation report to FDA, whether look-back notifications or recalls are necessary, and, if a device failure was involved, whether a medical device report (MDR) was required in accordance with 21 CFR 803.<sup>25</sup> Likewise, if an investigation is not conducted, the rationale for not doing so and the identification of the individual responsible for that decision should be documented.

It is essential that the records extend to the training and competency of the personnel responsible for these decisions; that is, it is a cGMP requirement that only trained personnel be allowed to make these judgments. Important assignments such as these reporting functions should be identified in the position descriptions for the personnel. Meticulous records of compliance with cGMP elements are required to assure both upper management and the FDA that an establishment is in control. Control, in this context, can be defined as compliance in every respect with all of the establishment manufacturing SOPs and FDA requirements. It is worth emphasizing that if a firm’s SOPs are more stringent than FDA requirements, it is not acceptable to deviate from the SOP, even if the FDA requirement is met, unless appropriate change control and reporting to FDA are carried out before the deviation. During inspections of blood centers, whether by the private sector or regulatory bodies such as the FDA, the overriding investigational concern is whether an establishment is in control. Records should be designed to document control of manufacturing systems.

Record-keeping is greatly facilitated by blood center computerization. Increasingly, information management and technology are being used to maintain batch records, document process control, and similar record-keeping activities. Validation (see below) of computer systems used to maintain blood center records is the subject for extensive and detailed guidelines published by the FDA.<sup>26-31</sup> Further, the FDA notified vendors of blood establishment computer software that these programs are medical devices subject to regulation [including 510(k) clearance processes], and blood centers must ensure that software used for control functions is a properly validated, 510(k)-cleared device, or equivalent.<sup>32-36</sup> In the final section of this chapter, the



application of information technology to improve process control through process innovation is discussed further.

### Medical Devices (§820)

The Center for Biologics Evaluation and Research (CBER) maintains a list of all blood establishment computer software (BECS) it has cleared (<http://www.fda.gov/cber/products/510ksoft.htm>). Although this list is a valuable resource for blood establishments that are adopting their first or a new BECS, it should not be the only resource used. Newly cleared BECS are continually added to the list, but those that are obsolete are not removed from the list. Furthermore, although the list includes the version of the software that was reviewed and cleared, it does not necessarily reflect the most current version available. Not all changes to a device, including software, require submission to and clearance by the FDA; thus newer versions not appearing on the list may be legally available. Most important, the limitations of the 510(k) clearance process must be recognized.

The 510(k) review process was implemented in 1976 as part of the Medical Device Amendments to the FD&C Act. In general, before 1976, as long as a device was not associated with adverse events it was allowed to remain on the market. Those that were associated with adverse events could be ordered off of the market and if they remained on the market were considered illegal. When the Medical Device Amendments were enacted, rather than call for submissions to the FDA for the countless number of devices already on the market, it was determined that any device that was legally on the market was safe by the virtue of the fact that it had not been ordered off of the market.

However, the Amendments required that these devices be classified so approximately 1700 different generic types of devices were identified and grouped into 19 medical specialties. The devices were classified into three classes based on the level of control thought necessary to ensure device safety and effectiveness. Class I devices are those that FDA determined needed the lowest level of control to ensure safety and effectiveness. Class II devices are those that FDA determined need to conform to performance standards in order to ensure safety and effectiveness. Class III devices are those that FDA determined need the highest level of regulation to ensure safety and effectiveness.

Two processes for bringing new devices to market were developed; the premarket *notification* submission as described in Section 510(k) of the Act and a premarket *application* as described in Section 515. The premarket notification submission became known as a 510(k), whereas the premarket approval application became known as a PMA.

Under a 510(k), the submitter is required to show substantial equivalence to a legally marketed device. A legally marketed device is one that was legally on the market before 1976 (a pre-amendment device) or has been shown to be substantially equivalent to another device that has been cleared through the 510(k) process. These legally marketed devices are known as the “predicate.” Under a PMA, the submitter is required to provide a reasonable assurance of safety and effectiveness.

BECS is reviewed under the 510(k) process and thus the review focuses on a determination of substantial equivalence. A substantial equivalence determination is based on the similarity of devices to one another but does not require that a new device be exactly the same as the predicate. This is an important concept for users to understand; all BECS do not have the same features and perform the same functions. It is the user’s responsibility to determine what the requirements are and to determine which device or devices meet those requirements.

Another important aspect of the 510(k) review process is the fact that it does not consider compliance of a device manufacturer or a device with other parts of the regulations. For example, except in a few cases, it does not consider whether the manufacturer is in compliance with the medical device quality system regulation (21 CFR Part 820), MDR requirements (21 CFR Part 803), electronic records and signatures (21 CFR Part 11), etc. All of these can have an impact on the ability of the product to meet the users’ needs and requirements but FDA has not reviewed them as part of the premarket submission.

With this understanding of what the 510(k) review process is and is not, the user needs to take an active role in the identification and selection of a BECS. As mentioned earlier in this section, the list of cleared BECS is additive and obsolete versions of software are not removed from the list. Thus, the mere presence of a particular BECS or version of a BECS on the list does not mean that it is available or that it will meet current user requirements. Likewise, the absence of a particular version of a BECS does not mean that it is illegal for the vendor to market it. If the version number being offered for sale is only incrementally larger than one on the FDA’s list, it is likely that the changes were minor and did not trigger the need for a new 510(k). To determine if the product is marketed under 21 CFR 820, the user can look into the manufacturer’s inspection history. Although the FDA does not post a list of those manufacturers with an acceptable compliance history, it does post information about manufacturers who have compliance issues (<http://www.fda.gov/ora/>). Other information that may be related to device performance is available in the enforcement report (<http://www.fda.gov/opacom/Enforce.html>) for recalls and at <http://www.fda.gov/cdrh/databases.html> for MDR reports. However, all of this up-front research does not eliminate the need for the user to perform adequate validation of the software before putting it into actual use.

Why is validation necessary if the manufacturer has performed validation studies and the FDA has cleared a device? Validation is required because the manufacturer cannot anticipate every configuration in which its device may be used and thus cannot test every configuration. Nor can the manufacturer anticipate and test every data entry error that may result in an unintended consequence. Likewise, the environment in which the software will be used will vary from location to location. Proximity of other equipment and wireless systems will not have a direct impact on the software, but can have an impact on the hardware the software is used with. Therefore, the user must validate the software in the context of the complete system, ie, in its

operating environment. Thus, as with any validation project, the software validation must be performed on-site with the users' hardware and other equipment, personnel, SOPs, etc. In fact, 21 CFR 211.68 includes computers as equipment needing validation. It is important to note that if the manufacturer indicates that the software is compliant with 21 CFR Part 11, the user needs to include validation of this aspect as well.

### Electronic Records (Part 11)

Part 11 was promulgated to describe the criteria under which the FDA would consider electronic records, electronic signatures, and handwritten signatures executed to electronic records to be trustworthy, reliable, and generally equivalent to paper records and handwritten signatures executed on paper. Where electronic signatures and their associated electronic records meet the requirements of Part 11, the agency will consider the electronic signatures to be equivalent to full handwritten signatures, initials, and other general signings that the regulations require. Electronic records that meet the requirements of Part 11 may be used in lieu of paper records, in accordance with 21 CFR 11.2, unless paper records are specifically required. As one would expect, the computer systems (including hardware and software), controls, and attendant documentation maintained under Part 11 are subject to FDA inspection and must be readily available.

There are many aspects to Part 11 and an establishment can choose which records are going to be kept electronically. There are important distinctions regarding open vs closed systems and the various types of records and signatures required. Thus, when a facility is considering use of electronic records or signatures it is particularly important for the definitions in 21 CFR 11.3 to be understood.

Other software products, such as programs used for logging and analyzing data trends from quality system activities or from complaint and MDR systems, are available for use in blood establishments. Although these other types of software are not medical devices, they can be considered equipment and should still be validated for their intended use because they can have an indirect impact on product manufacturing. As an example, one facility had a calibration scheduling program that calculated the date of the next calibration in a manner different from that in the firm's SOP. This resulted in the calibration expiring before the recalibration date calculated by the software. The firm was deemed to be manufacturing outside of the SOPs and using equipment that was not calibrated.

### Personnel and Training (§§606.20, 211.22 through 211.34)

The requirements of the personnel regulations are designed to ensure that people engaged in the manufacture of biologics and drugs have sufficient education, training, and experience to perform their assigned functions. Section 211.22 also requires that employees be trained in cGMP by qualified individuals. Although these provisions present only the most general requirements, it is recognized that several key components must be included as part

of a personnel training program that complies with cGMP and that ensures SOPs are correctly implemented by all staff.

### Training

Training should be performed formally by personnel with documented credentials attesting to their qualifications, and the records of training should include clearly defined objectives and course content. Training in general systems (eg, computers, incident reporting, safety) are as important as technical training specific for an employee's tasks. Pre- and post-training examinations are one acceptable way of documenting the attendance of the employee and the effectiveness of the training. Refresher courses must be offered at predetermined appropriate frequencies and whenever a manufacturing or process control is changed. It is also critical that experience, education, and competency of staff and contractors be documented, without regard to whether they perform maintenance and repairs, training, auditing, or other staff functions, and particularly if they are expected to perform with little additional training after hire. In the absence of this training documentation and certification of credentials, it is critical that tasks performed be directly supervised; for example, records of laboratory equipment repair should be countersigned by the laboratory staff before the repair technician leaves the premises. It must be understood that the blood center remains responsible for all work performed on its behalf and the countersignature represents an assessment not only that the technician was present on a given day but that the work listed on the work order was in fact performed.

### Competency Testing

In addition to evaluations demonstrating effectiveness (pre- and post-training testing) accompanying formal training, personnel should demonstrate continued competency by periodic examination, review of records they generate, and actual task performance. The results of these assessments should be retained by the quality unit as well as part of employee training records. Personnel records should be subdivided to permit easy access to the training portions (and the corrective action relative to errors, if applicable) without divulging any confidential portion of the personnel record. If a weakness is detected, retraining and reevaluation become critical corrective actions. An employee who fails a competency evaluation (or makes a serious error in performance of duties) must not continue to perform that task without documented supervision until retraining and a satisfactory evaluation is recorded. The content of the assessment exercises is also a required record. It is not acceptable practice to discard the tests and maintain only the scores; however, maintaining a single master file copy of the test (rather than every employee's copy) is acceptable as long as scores can be related to the appropriate test instrument.

Some employees may feel threatened by these activities; however, they can be approached constructively. Proper training improves the performances of all employees and they should be taught that they are better prepared to comply with cGMP through training. Employee acceptance can be improved by

**Table 64-5.** Calibration Deficiencies: Synopsis of Warning Letter Citations

- 
- Written procedures for record keeping and equipment calibration are not followed.
  - Calibration logs, travel tickets, and donor files contained illegible corrections.
  - No documentation was available showing calibration of all equipment such as the donor scale, refrigerator alarm system, trip-scale temperature gauges, and thermometers used to monitor the incubator and freezer.
  - Records maintained for the incubator, freezer, and hematocrit analyzer revealed several instances of the equipment temperatures or calibration being outside acceptable ranges; no documentation was available to show that deviations were investigated or that corrective actions were implemented.
  - Equipment was not calibrated as prescribed in the standard operating procedures (SOPs) (21 CFR 606.60) in that the rpm calibration and timer check of the centrifuge was not performed every 60 days as required in the written procedure and operator's manual; vital signs monitor was not calibrated every 6 months as required.
  - An employee failed to enter the reference pipette calibration constant as required by the firm's SOP.
  - The SOP for microliter pipette calibration does not require a time frame for review of the calibration records; as a result, review of records did not occur until 6 to 14 months later.
  - The apheresis department does not have an SOP requiring a time-frame for review of equipment maintenance and calibration.
  - No document is available to show calibration and maintenance of the recording thermometers.
  - Quality control checks on other laboratory equipment are not being performed in accordance with established written procedures.
  - Numerous records reviewed during the inspection contained illegible corrections, and there is no documentation that these records were reviewed by a supervisor.
  - Staff failed to calibrate the stopwatch used in the calibration of the Hematastat's timer, the tachometer used to calibrate the Hematastat's rpms, and the sphygmomanometers.
  - Established written procedures for apheresis equipment calibration, monthly maintenance, and cleaning to avoid possible cross-contamination are not being followed.
  - There are no quality control and calibration records for the Diatek donor thermometer and the blood pressure cuff.
  - There are no quality control and calibration records, nor is there a written procedure for the quality control and calibration, for the scale used for collecting blood.
  - There were incomplete records of the quarterly performance testing of automatic cell washers and quarterly calibration checks of the Coulter counter instrument as required by the blood bank's written SOPs.
  - Records showed deficiencies related to the secondary review of analytic and calibration data.
- 

CFR = Code of Federal Regulations; rpm = revolutions per minute.

emphasizing that the performance of each employee is critical to the organization's success in complying with cGMP to ensure safe products, and that cGMP compliance is vital to the overall quality and regulatory compliance of blood center operations.

### Retraining

Ad hoc training of employees who have experienced lapses and made errors forms a vital step in continuous improvement programs (see below). When there is a documented event that requires retraining, such as an error that is detected after a product has been released or an employee who has failed a competency evaluation or proficiency testing, the retraining and its effectiveness must be documented as with other forms of training. However, citing retraining as the primary corrective action for an error raises additional questions. If this person's competency evaluation had inaccurately predicted the ability to perform, are there others failing to perform? Was the original training adequate or does the basic training need revision? What products may have been affected by inadequately trained staff? Is supervision of the employee adequate if the deficient performance was not detected until an error was made?

### Calibration (§§606.60 and 211.68)

Calibration has been defined as the comparison of a firm's measurement systems with known standards. An example may be helpful in describing calibration requirements. A centrifuge used to manufacture platelets has multiple process points that can be calibrated. Among these are the centrifugation timer, its tachometer,

and its brake timing. Subpart H (Laboratory Controls) of 21 CFR 606 outlines calibration requirements for several common devices used in blood collection, processing, and testing. Section 211.160(b)(4) outlines specific calibration provisions, requiring that all "instruments, apparatus, gauges, and recording devices must be calibrated under established protocols that specify periodicity." These provisions are applied to blood centers, and there is increasing evidence that FDA will apply all requirements stringently. Examples of deficiency citations that relate to calibration issues are found in Table 64-5. Other areas that some centers fail to address are the qualification of the equipment that will be used in calibration and the qualification of procedures and staff used by contractors performing calibration services. Blood centers retain full responsibility for ensuring that work will meet FDA requirements, whether it is done in-house or contracted.

In the 21 CFR 600 series, under Subpart D (Equipment), it is required that all production equipment be calibrated "... on a regularly scheduled basis as prescribed in the Standard Operating Procedures Manual." In Section 606.60 the calibration requirements of common blood bank equipment are provided in tabular form. This table is incomplete, however, and many of the automated systems currently used in blood centers are not listed. Guidance then must be sought in Section 211.68 and the manufacturer's instructions for the item.<sup>37</sup>

As noted, blood centers must have a calibration SOP that specifies the center's policies regarding this requirement. Traditionally, blood centers have delegated responsibility for equipment calibration to the section in which the equipment is used. A more

comprehensive approach is frequently used in the pharmaceutical industry in which a metrology unit is responsible for calibrating all equipment in the establishment. Scheduling can be computerized and can be integrated with process innovation system designs (see below). Centralized calibration of blood center measurement equipment provides several advantages, whether the metrology functions are provided by full- or part-time personnel, and is the preferred organizational approach. Management can easily review the calibration efforts of the establishment by consulting with one unit and one computer printout, centralized file, or set of records.

### Validation (§211.68)

Validation has been defined as documented evidence that provides a high degree of assurance that a specific process (or product) will consistently produce an outcome that meets established quality and performance specifications. Despite the lack of detailed validation requirements in either the 21 CFR 200 or 600 series, the FDA has focused on validation (especially of computer/software systems) during center inspections, at workshops, and in guidelines and memoranda.<sup>19,26-31</sup> It is clear that as blood bank systems continue to increase in complexity, validation of these systems will continue to be an important element of cGMP. During cGMP training, calibration frequently is confused with validation. Calibration deals with the assurance that critical measurement instruments for specific pieces of equipment used in the manufacture of blood components measure accurately.

Several aspects of validation are recognized as critical: system descriptions (including validation plans and protocols with established criteria for pass and fail), a defined process for review and acceptance of data including procedures for tracking and resolving all discrepancies, allocation of responsibility for the go ahead or no go decisions, assessment of impact on operations and revalidation requirements.

### System Description

Validation of a system cannot be achieved without an accurate description of the process that is to be validated. The description of the system should contain identification of all major pieces of equipment, software, hardware, and flow diagrams, as well as verbal characterizations of the systems or processes. Acceptance/rejection criteria should be described whenever appropriate. This aspect of validation is of particular importance for computer systems for which, as noted, specific validation guidelines have been published by the FDA.<sup>26-31,38</sup>

### Validation Protocol

A key component of validation, whether retrospective or prospective, rests in developing and executing a validation protocol and planning its integration with operational systems. This protocol should prospectively specify the manner and documentation of system or process validation, the testing to be performed (including stress or boundary testing), and acceptable statistical outcomes. Test protocols should address all operational situations and all equipment or hardware that will be used, all sites, minimal and maximal

load conditions, stress, interference, boundary conditions, etc, that can reasonably be anticipated. The protocol should include the validation of the physical environment of the system (installation qualification, or IQ), the testing of the equipment as installed (operational qualification, or OQ), and the plan to follow up on the actual operation of the system by trained staff after integration in routine usage (performance qualification, or PQ).<sup>29,38</sup> It should be noted that although retrospective validation may provide valuable information and some level of confidence in the object being validated, it should not be used in lieu of adequate prospective validation.

### Revalidation Requirements

Protocols should specify when system and process revalidation should be undertaken. Revalidation is integrally linked to both process control (see below) and change control. The latter should be governed by change control SOPs.

Historically, validation requirements for blood centers developed bidirectionally. Blood centers explored what was doable for their legacy systems, and the FDA defined best methods for validation of blood center systems in general, enforcing validation requirements when failure to validate resulted in operational errors. The private sector offered language that would extensively revise the proposed language but not change its effectiveness,<sup>39</sup> and the FDA to date has not finalized their draft guidance on blood bank systems, but relies on guidance issued for validation of computer systems for all FDA-related purposes.<sup>31</sup> On the other hand, the International Society of Blood Transfusion (ISBT) has issued guidance on validation of automated systems in blood banking.<sup>40</sup>

### Labeling (§§211.122–211.130; §606.120)

An important aspect of labeling of blood components lies in the definition of a lot or batch. In the 21 CFR 600 series, a lot is defined as "... having been thoroughly mixed in a single vessel." Clearly this definition relates only imprecisely to the production of blood components by blood centers and limits a *lot* to products from a single donor. Therefore, the term *batch* has more meaning in relation to release of products, and in the blood arena *batch* is usually defined as a group of products for which the laboratory tests were performed at the same time. Essentially, a batch release has the same meaning for blood centers as what the FDA has called a lot release. One of the difficulties created by these definitions rests in the inability to test each lot completely. This inability dictates that manufacturing controls must be in place to regulate product quality precisely, because only limited testing of the final product is practical. In many blood centers, labeling is used as a control point or final check that a unit, or lot, is suitable for transfusion or further manufacture, and that other required work, such as testing for viral markers, has been performed on the batch. Each component is checked, usually by a computer, to ensure that all of the testing and record checking has been completed and that no disqualifying information appears in the past or current records of either the donor or the donated unit. If the product is stored for any period after the initial release, there must be procedures to ensure that receipt of any postdonation information or

seroconversion of a donor results in appropriate interdiction of the products collected earlier, regardless of where they are stored.

It is essential that labels be recognized as a critical control element requiring strict procedures for inventory, archives, and change control, as well as appropriate checks of their acceptability for use (release to inventory) and approval of all changes. It is noteworthy that the recall provisions of 21 CFR 7.40-7.50 require a copy of reconstructed label(s) of the product being recalled to be provided to the FDA, regardless of the amount of time elapsed since the product was issued.

Increasingly, blood centers are installing automated label printers that make on-demand custom labels for components only after an electronic record review has found each suitable for release. These systems also permit electronic label reconciliation to approach label accountability requirements of 21 CFR Section 211.120. If duplicate labels can be printed, strict controls are required to avoid a mislabeling. Product release systems may include automated checks as additional safeguards at the time the shipments are packed. Two-dimensional barcoding permits encrypting a great deal of information about the unit on the label. The FDA has strongly encouraged transition to these systems through its support of the ISBT 128 barcodes and publication of guidance regarding its recognition of the standard. The agency has also cleared for marketing (<http://www.fda.gov/cber/efoi/510k.htm>) several transfusion safety management systems that use the barcode on the blood component unit in conjunction with a barcode on the recipient's wristband to ensure that the right unit is given to the right patient.<sup>41</sup> On-demand label printing should be validated to ensure that peculiarities of the printer(s) and labels used do not have an adverse effect on the final product labels. Printer technology has vastly improved, but the limitations of some printers, particularly dot matrix printers, make printing of labels difficult. In some cases, text is nearly impossible to read and does not allow for some of the font characteristics required in blood establishments. The most notable situation was related to the printing of reports rather than labels. Because the device printed only in capital letters, anti-C could not be differentiated from anti-c. This situation emphasizes the importance of validation of printers.

The FDA requires blood to have machine-readable labels in a format approved by the FDA [21 CFR 606.121(c)].<sup>13</sup> The information on the label that must be machine-readable includes:

- A unique facility identifier.
- Lot number relating to the donor ("unit number").
- Product code.
- ABO/Rh.

ISBT 128 is an international standard for the transfer of information pertaining to blood, tissue, and cellular therapy products that is already in use in many countries. It uses internationally agreed-upon terminology and label formats to ensure clear, unambiguous labeling. Use of common databases to interpret barcodes allows users to overcome language barriers when products are shipped internationally. Scanning of barcodes can serve to "translate" the language on the label. Features of ISBT 128 include:

- Donation identification numbers that are unique worldwide for a period of 100 years.

- A detailed product code database for blood, tissue, and cellular therapy products that is readily expandable.
- An international facility identification database.
- Improved process control capabilities through the use of standardized data identifiers, keyboard entry check characters, and concatenation of barcodes.

Information on ISBT 128 is available from the International Council for Commonality in Blood Bank Automation (ICCBBA), the organization that manages the standard (<http://www.iccbba.org>). The FDA provides guidance on barcode label requirements (<http://www.fda.gov/cber/gdlns/barcode.htm>).

### Error Management [21 CFR 211, §§606.160(b)(7)(iii) and 606.171 and 606.100(c)]

Other than the obligation to report adverse reactions as provided in 21 CFR Section 606.170 and the investigation of errors with documentation of corrective action as provided in Section 606.100(c), an integrated error management system is not specifically required by the provisions of Title 21, although the rules in 21 CFR 606.171 mandate the reporting within 45 days of all biologic product deviations on released products. Table 64-6 provides a summary of the reporting criteria and examples of reportable and nonreportable events. Traditionally, 21 CFR 600.14 has always required licensed facilities to report promptly any error that may affect the safety, purity, or potency of the product, and it was understood that a system was needed for capturing these events consistently. The FDA has recognized the importance of this cGMP element in a memorandum and regulations issued in 2001 (21 CFR 606.171). The rules are more broadly applicable to blood establishments than any preceding policies, although they are narrowly directed.<sup>42,43</sup>

The investigation and follow-up of deviations is critical to the successful improvement of blood center quality. Appropriate management of errors and accidents (or deviations in the current FDA terminology) forms the centerpiece of continuous improvement. Each blood center deviation should be treated as a "jewel" to be used as a case study for the staff to improve center processes. All facilities must develop SOPs for reporting, managing, and correcting errors and deviations. These SOPs should form the centerpiece of both blood center staff training and continuous improvement. Error management efforts and results should be documented in the records of the quality unit. Monitoring corrective action outcomes to ensure effectiveness is an essential part of the system for addressing corrections. Figure 64-4 illustrates a process diagram of error management; the similarity to the diagram of the prevention model of quality control (Fig 64-2) is evident.

Although error management systems are not defined in Title 21, some attributes of good systems have emerged both in blood centers and in industrial manufacturing establishments.

1. Employees at all levels in the organization are encouraged to report errors. Punitive policies discourage reporting and hence opportunities for systems improvements may be lost.
2. Employees involved in the process in which an error was made should be involved with the investigation and resolution of the error and process change. It is essential that the investigation

**Table 64-6.** Synopsis of Final Rule, on Reporting Biological Product Deviations<sup>2</sup>

- The rule applies to all establishments: donor centers, blood banks, transfusion services.
- Reporting time is not to exceed 45 days.
- Report by mail: Director, Office of Compliance and Biologics Quality, 1401 Rockville Pike, 200N (HFM-600), Rockville, MD 20853-1448; electronically: CBER's Web site: [www.fda.gov/cber/biodev/biodev.htm](http://www.fda.gov/cber/biodev/biodev.htm).
- If the answers to the questions below are affirmative, the event is **reportable**:  
 Was the event associated with manufacturing?  
 Did the deviation affect safety, purity, or potency?  
 Did it occur in a licensee's or a contract facility?  
 Did the facility have control over the product when the deviation occurred?  
 Was the product distributed?

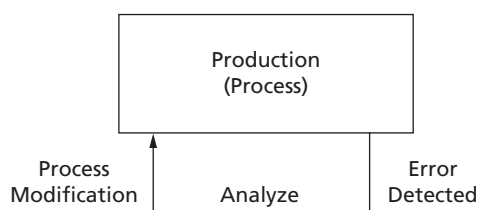
**Reportable Biologic Product Deviations**

- An event occurs that may affect product purity, safety, or potency.
- Postdonation information is received that potentially affects safety.
- Compatibility sample was collected from the wrong patient.
- Collection or processing materials did not meet specifications.
- FDA requirements were not met on donor deferral and screening.
- Current or subsequent viral marker test was positive.
- Donor screening (history, arm inspection, etc) was not recorded.
- Both confidential unit exclusion stickers were not applied to unit.
- Donor deferral lists were incorrectly checked.
- Bag used for collection was outdated.
- Donor sample of unit was clotted or hemolyzed.
- SOPs for collections were inadequate or not followed.
- Collection time was not documented or extended.
- Time frame for collection or processing was incorrect.
- Use of special procedures was inappropriate.
- Testing was not performed in accordance with instructions.
- Unsuitable samples were used for testing.
- Mislabeling of ABO and the like occurred.
- Storage or shipping temperatures were incorrect.
- Unsuitable units were not quarantined.
- Special orders were not filled (for example, cytomegalovirus-negative).

**Examples of Nonreportable Biologic Product Deviations**

- Transfusion errors occurred outside the blood facility.
- An error was corrected before distribution and safety was not affected.
- Look-back, retrieval, or notification procedures were not followed.
- Donor protective measures were not met (age, colds, flu, etc).
- Previous donation records were not checked.
- Labeling errors occurred that did not affect safety (short expiration, etc).
- Shipping paperwork contained errors.
- Units were returned because of temperature deviations.

FDA = Food and Drug Administration; SOPs = standard operating procedures.

**Figure 64-4.** Error management process. Note similarity to the prevention model of quality control diagrammed in Fig 64-2.

be sufficient to determine the underlying root cause for the error rather than identifying mere “symptoms” of the root cause. The FDA also requires that staff be educated concerning the effect their errors have or potentially have on product quality; that is, it is required that they understand how their responsibilities have an effect on ensuring that only safe products are released.

3. Confirmation of the effectiveness of the corrective action is vital to ensure that the improved process continues to yield the expected results. Post-change monitoring is essential and long-term evaluation should be undertaken at appropriate intervals, eg, 3 or 6 months after a process improvement is completed.

**Quality Control Unit and Internal Audits [§§600.10, 606.20, 211.22, 211.192, 211.180(e)]**

Meeting the quality system provisions of Title 21 facilitates compliance with cGMP. As noted, Section 600.10 provides that an authorized official must be appointed to exercise control over all matters relating to compliance. Further, this designee must understand the scientific principles of manufacture. No similar provision is found in the 21 CFR 200 series, although the designated “official correspondent” is probably an equivalent function. A licensed establishment must tell the FDA who has been designated as its authorized official(s) and must report any changes. Similarly, an unlicensed registered facility must identify a reporting official on FDA Form 2830 as part of its annual blood establishment registration. The rationale for these provisions rests on the need for the agency to be able to deal with a responsible contact person in an establishment. Although the FDA permits the designation of alternative authorized officials, it takes no responsibility for the communication that must occur within a company. If a firm designates multiple authorized officials, the FDA may communicate with any one of them and the company must ensure that the information is disseminated appropriately. The authorized official can commit the establishment to certain courses of action, such as recalls and market withdrawals. Case law holds that upper management cannot avoid adverse consequences of noncompliance with regulatory requirements because a lower-ranking employee is designated the authorized official.<sup>44</sup> Some experts believe that the chief executive officer should be the responsible head (authorized official) to indicate the commitment of senior management to the principles of cGMP.<sup>45</sup> The Guideline for Quality Assurance in Blood Establishments states that, “A QC/QA Unit should report to management or its designee. In licensed firms, this unit should report to the Responsible Head who is the individual designated in the establishment license application to represent the firm in its regulatory activities with CBER [See 21 CFR 600.10(a) . . . ].”<sup>19</sup> However, this reporting structure is not wise unless regulatory compliance is a genuine part of the direct responsibilities and focus of this individual.

Section 211.22 states that an establishment must have a quality control unit [QC unit, quality program, or quality assurance (QA) staff] with several responsibilities designated in the CFR. Table 64-4 lists the responsibilities of the unit, including the authority to approve or reject all components and the approval

**Table 64-7.** Key Elements of a Blood Center Audit Program

|                                                                                                                                                                                                                                                                                                                                                                                           |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <ul style="list-style-type: none"><li>• Written audit protocols.</li><li>• Audits of defined scope and purpose.</li><li>• Knowledgeable audit team members.</li><li>• Review of audit reports by authorized official.</li><li>• Referral of adverse finding for error management.</li><li>• Audit teams independent from production.</li><li>• Scheduled follow-up and reaudit.</li></ul> |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

of all written procedures used in the establishment. Although not required by the CFR, it is generally held that the QC unit should organizationally be distinct from manufacturing to ensure its independence, and this de facto requirement appears in FDA guidelines.<sup>19,46</sup> In many blood centers the director of the QA unit reports to the chief executive officer.

Although internal audits are also not specifically required by regulations, procedures to detect problems are required and internal audits are important to ensure that processes remain in control (see Production and Process Controls). Audits are included in the quality control guidelines and reference is made to Section 211.180(e), which requires written procedures for evaluation of manufacturing records. Audits, whether performed by the facility staff, by customers, or by contracted third parties, can serve a more substantive role in detecting manufacturing problems. The key elements of an audit program are listed in Table 64-7. Integration of audits with error management offers an opportunity to augment continuous improvement efforts of blood centers proactively, the result of searching for errors and defects.

The quality control guidelines in effect since 1995 (and very similar to the 1991 and 1993 versions) suggest that audits focus on the adequacy of the critical control points outlined in the guidelines.<sup>19</sup> One useful approach employs audits to assess the completeness of compliance with a cGMP element throughout the facility. For example, training and competency records could be reviewed in each unit throughout the center. In a second approach, a specific process can be audited and each skill box evaluated for compliance with all of the cGMP elements. Centers should develop audit procedures adaptable to their own operations.

Internal audits are considered confidential by the FDA and not available to FDA investigators unless fraud is suspected or there appears to be an imminent threat to public health. This policy is in place because if audit reports were reviewable by the FDA, reporting of audit findings might be discouraged. The FDA also respects supplier audits as confidential internal audit information. However, it is critical that the center have readily available SOPs for audits, schedules demonstrating that all elements of operations are reviewed regularly (at least annually), processes for ensuring and documenting corrective and preventive action and follow-up, and formal closure notices to complete the records. It is also essential that any deviations discovered in the course of audits be properly documented in records that are available to FDA investigators.

Quality units can be deployed to enhance the quality of blood center operations in a variety of ways. They can assume

responsibility for personnel training, especially in cGMP, maintain calibration and validation records, design validation protocols, identify trends through statistical analysis, etc. A well-directed quality unit can be of enormous value to both the authorized officials and the staff of the facilities in which it is established. However, quality unit staff are not the ideal staff to write SOPs, because they need to be the final approval authority and their objectivity cannot be ensured when reviewing their own work.

**Facilities and Equipment (Multiple Sections of Both the 600 and 200 Series)**

Most of the provisions in both the 200 and 600 series deal with sufficient space and work flow design to prevent mix-ups and, in the case of interviewing blood donors, ensure privacy. Written procedures, schedules, and methods for maintenance and cleaning are also addressed. To meet cGMP requirements, the contracts for cleaning, pest control, and maintenance must specify the materials to be used, the schedules, and the procedures, and must show evidence of review and acceptance by the contracting facility. Such records must be reviewed by the firm periodically, and should meet cGMP documentation standards with dates and initials, no cross-outs, etc. Signature logs should show inclusive dates of employment. Training documentation for all of the tasks performed, including avoidance of biohazards, cGMP and documentation practices must be available and show evidence of review by the quality unit staff.

The segregation of unrelated activities and the preservation of clean and dirty pathways for both in-process materials and staff are basic cGMP principles. Section 606.40 emphasizes quarantine facilities, because it is critical to physically separate components unsuitable for release from those suitable for transfusion or further manufacture. Sterile supplies should be protected from inadvertent exposure to dirty conditions or biohazard waste. Incoming goods must be physically separated from released-for-use inventory, and supplies with specific temperature requirements during storage (eg, copper sulfate and plastic blood containers) must go promptly to monitored areas. All records must be protected against inadvertent loss or alteration, which translates to well-organized, locked storage or restricted-access areas. The FDA has defined prompt retrieval of records to mean “availability within a few hours.” Off-site storage of blood products by licensed facilities requires pre-approval (license supplement) by the FDA and close oversight by the blood center to fulfill its responsibility to ensure that all FDA requirements will be met. Schedules of performance checks, as well as calibration, are provided in a table in Section 606.60, but as with calibration requirements, much of the equipment used today is not specified in this table. Centers must develop their own schedules. In Section 606.65 frequency of serologic reagent checks is specified.

**Process and Production Controls [§§606.100, 606.140, 211.100, 211.101(c)]**

In a sense, all of the cGMP elements are embodied in production and process controls. The requirements for SOPs, calibration, etc, are outlined in these provisions, as are a variety of quality control

procedures. The FDA accepts SOPs as provided by manuals of the AABB and the American Red Cross [§606.100(d)]. However, when these are referenced, it is important that the facility identify exactly which parts apply and determine that there is no conflict with manufacturer's directions or other FDA requirements. As previously emphasized, the determination that an establishment is in control is the most important attribute in the preservation of an establishment license.

With respect to validation testing, the reagents themselves are not being validated but rather the test *system* is being validated. Users should validate any new test before using it to test actual samples and should revalidate anytime a significant change to the system occurs. The underlying concepts for validating a test system are the same as for process and product validations. The components of the system should be listed and adequately described. Validation should address factors within the user's control at both the minimum and maximum criteria. For example, if the package insert for a reagent gives a range for incubation temperature, incubation time, centrifugation time, centrifugation speed, etc, the test system should be evaluated using both the minimum and maximum parameters. Likewise, if a test kit can be used with different brands or models of instruments, validation should be conducted for each instrument in the facility.

With regard to acceptance testing and routine QC testing, these tests are needed because once the reagent leaves the manufacturer's storage, it is no longer in its control. Although the FDA does require stability studies and shipping studies, among others, in support of licensure, the manufacturer cannot anticipate all of the adverse circumstances the reagent may be exposed to in shipping, use, and subsequent storage and therefore cannot test under them. The fact that most reagents used in blood establishments are derived from biological materials, which are labile and sensitive to many external factors, further complicates the issue of performance and acceptability.

Thus, the purpose of acceptance testing is not to test the manufacturer's ability to ensure lot-to-lot consistency but rather to determine if the reagent was exposed to and affected by adverse shipping conditions such as extreme heat or cold.

Once the reagent performance has been deemed acceptable for putting the reagent into use, it must be subjected to QC testing throughout its use. Quality control testing serves to evaluate the use and storage of the reagent. Most of the reagents are multi-use products opened and entered repeatedly over a period of days or even months. Testing should be directed at detecting product deterioration caused by contamination with other reagents or microbes, improper storage and use temperatures, excessive mixing or vibration, etc. It can also serve as a means of monitoring equipment function and user proficiency. For some reagents (eg, immunohematology reagents), minimum criteria for performance of QC are described in Title 21. For reagents for which there are no QC requirements in Title 21, the QC testing described in the manufacturer's package insert must be followed. In laboratories where clinical samples are tested, Clinical Laboratory Improvement Amendments (CLIA) requirements for QC, listed at 42 CFR 493, must also be followed.

Not all reagents used in blood establishments need to be licensed. Title 21 CFR 610.40 requires the use of FDA-approved test kits for the performance of tests for communicable disease agents. The agency provides a list of currently licensed human immunodeficiency virus (HIV) and hepatitis test kits (<http://www.fda.gov/cber/products/testkits.htm>). Title 21 CFR 640.5 requires licensed "or equivalent" anti-A, anti-B, and anti-D for blood typing but the regulations do not require that other immunohematology reagents be licensed. The expectation is that if licensed reagents are available, blood establishments will use them. If a licensed reagent is not available, reagents and tests developed in-house (eg, home-brew reagents), or expired licensed reagents may be used providing appropriate controls and labeling are in place. One example is when source material for a particular blood grouping reagent is no longer available or of sufficient reactivity to use as a reagent. In these cases, blood establishments may choose to use expired reagents with appropriate controls or serum from patients or donors known to contain the subject antibody. The blood establishment should use only those expired reagents or sera/plasma in an emergency with the approval of the medical director, and only when appropriately tested with positive and negative control cells. Also, if the blood establishment uses unlicensed sera/plasma to type a patient, it should retype the patient with licensed reagents when they are available. If the blood establishment uses unlicensed sera/plasma routinely, it must meet the requirements in 21 CFR 660.25 and 660.26. The facility must have an adequate QC program to monitor such sera/plasma. If the blood establishment uses unlicensed sera/plasma to type donor units, it should notify the consignee by attaching a label to the unit with the statement "Tested and found negative for XX-antigen using unlicensed typing reagents" or an equivalent statement. The establishment may use a tie-tag attached to the unit for the additional labeling. The FDA "Compliance Program Guidance Manual; Chapter 42 - Blood and Blood Products; Inspection of Licensed and Unlicensed Blood Banks, Brokers, Reference Laboratories, and Contractors 7342.001," provides more information on controls and labeling.

Most recently, some of the larger blood establishments have developed or are developing molecular methods for in-house use. These facilities generally have developed test methods to help them provide Red Blood Cell units with extended red cell typing for transfusion to patients with multiple antibodies or to reduce exposure to foreign antigens in transfusion-dependent patients. Although molecular tests have been available in some countries for many years, there are currently no licensed/approved/cleared molecular tests for blood typing available in the United States. It is expected that once methods are developed that allow for high throughput in a short period, there will be more interest in this technology and products will be submitted to the FDA for premarket clearance or approval. In anticipation of these future submissions, the FDA cosponsored a workshop with the National Institutes of Health.<sup>47</sup> In the meantime, several protocols are available for other medical disciplines that might be of interest (<http://www.clsi.org/>).



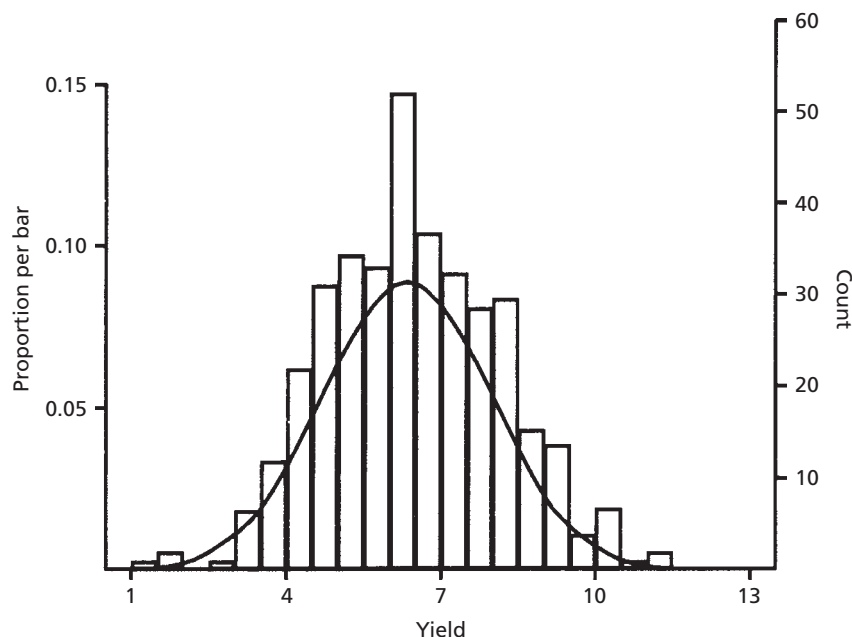


**Figure 64-5.** Platelet yield distribution. (Used with permission from Zuck.<sup>50</sup>)

## Current Good Manufacturing Practice Violations

The FDA periodically publishes common violations of cGMP. The classification of deficiencies cited on FDA 483 forms from routine blood center inspections indicate that many of these violations fell into four broad categories.<sup>18</sup>

- 1. Donor deferral.** Deferral records were incomplete and multiple reasons for deferral were not recognized. Incomplete or inaccurate donor identification was noted as a recurring problem.
- 2. Viral testing and review of testing records.** A variety of failures were outlined, including poor sample identification, failure to follow manufacturer's instructions, improperly qualified equipment, and samples tested numerous times without proper initial test invalidation (so-called *testing into compliance*).
- 3. Computer systems.** Failure to validate computer systems and lack of documentation of computer training and validation of change procedures were cited frequently.
- 4. Standard operating procedures and control of operations.** Citations included lack of adequately detailed SOPs for all steps in manufacture and/or failure to follow the SOPs.

A more recent and comprehensive "top 10" list released by the agency reflects all types of FDA-regulated facilities and is shown in Appendix 64-1.

## Process Control Imperative

Complying with cGMP and correcting deficiencies only begin to meet the public expectations for quality performance in manufacturing and distributing the nation's blood supply. Blood and plasma centers are developing quality improvement programs

based on principles of continuous improvement, statistical process controls (SPC), and process innovation. The objective of these efforts is to reduce lot-to-lot variation in blood component manufacture and the risks of blood transfusion to as low as reasonably achievable, a concept that originated in the reduction of risks associated with exposure to ionizing radiation.<sup>48</sup>

Continuous improvement initiatives are centered on error management by redesigning processes to prevent an error from recurring. They also embrace the prevention model of quality management that is rooted in SPC. As represented in Fig 64-2, this model monitors the various steps in manufacturing in an effort to determine which steps are, and which are not, in control. The many tools available to assist in these activities are beyond the scope of this chapter. However, several of the more important ones relate directly to cGMP compliance—frequency histograms (Pareto charts), Shewhart control charts (also called  $\bar{x}$ ,  $r$  charts), and process capability studies.<sup>49</sup>

Histograms are useful in plotting the results of individual process outcomes and to prioritize corrective action. Figure 64-5 illustrates a histogram describing results of platelet concentrate manufacturing at one center. The Gaussian distribution of this histogram, provided it is similarly contoured and placed as on prior charts, suggests that the manufacturing processes are in control. A kurtotic or skewed distribution or a shift in the mean count to the right or left would suggest that processes may not be in control.

Shewhart or control charts are regarded as a singularly important tool of SPC to detect drift and variation in manufacturing processes.<sup>49</sup> They permit identification of process weaknesses that result in lot-to-lot variation. When identified, process redesign can be undertaken to improve manufacturing and product quality.

Pareto charts offer another tool for blood center quality improvement.<sup>49</sup> The frequency of cGMP problems discovered by audits or error management are charted on a histogram and give management the option of addressing first those problems that have both the most frequent occurrence and the highest likelihood of causing release of blood components unsuitable for transfusion. One of the difficulties of the current FDA inspection approach lies in its inability to differentiate a major from a minor infraction. For example, on Form FDA 483, failure to perform specific gravity of a copper sulfate solution may appear as a cGMP violation of equal importance to performing faulty HIV donor screening. In the United Kingdom, inspectional findings of blood centers are graded from critical (those that pose an imminent health hazard) to other (those of low probability of causing damage). This strategy permits both the inspected establishment and the public to assess the seriousness of the findings. The FDA has established grades of license change applications, and favors risk-based assessment of the threat to public health posed by deficiencies. Blood center personnel are encouraged to focus on those deficiencies most likely to affect the public health. Meanwhile, Pareto charting as a tool to determine priorities for error management within a blood center will partially compensate for inspection and recall policies that may not reflect the seriousness of deficiencies.

## Future Initiatives

### Process Innovation

Implementing cGMP represents only the initial effort to improve the safety of the blood supply by strengthening process controls in blood centers. Process innovation presents another opportunity for centers to improve significantly the safety of the blood supply.<sup>51</sup> To oversimplify, process innovation employs information technology (IT) to control manufacturing processes.<sup>23,51</sup> Recent initiatives have included work known as process and analytical technology (PAT), which is defined as systems for analysis and control of manufacturing processes based on timely measurements, during processing, of critical quality parameters and performance attributes to ensure an acceptable end product. This work is based on the premise that all products have critical quality attributes, and process variables can be controlled to maintain the critical quality attributes within acceptable limits. PATs are applied to achieve both understanding and control of process variables that are causally linked to product quality attributes.

Initiated in industry to improve productivity, process innovation is being instituted in blood centers to improve quality and is defined in this chapter as the prevention of the release of units unsuitable for transfusion. Integrating computer control of manufacturing processes offers a significant advance to reduce further the risk of infectious disease transmission by blood transfusions. A few examples may illustrate the power of this strategy. Currently, blood center computer systems can be configured to prevent recruitment of deferred donors. Because deferred donors have been excluded from the rolls presented to telerecruitment

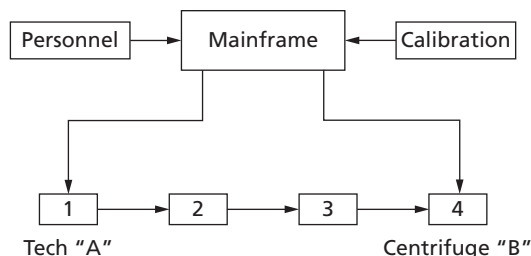


**Figure 64-6.** Theoretic process innovation in blood centers.

departments, they cannot be recruited. This principle can be extended. Figure 64-6 illustrates the capability of IT to control manufacturing processes. Personnel records can be linked with production records to prevent unsuitable employees from using their access codes to create batch records. Similarly, a centrifuge in need of calibration on a certain date could be precluded from use beyond the calibration expiration date. The power of linking IT with process controls lies in preventing the manufacture of components unsuitable for transfusion. Components not manufactured cannot be released. Other examples of process innovation designed to increase the safety of the blood supply can be envisioned. More advanced systems will emerge as this technology is implemented in blood centers throughout the country. As discussed previously, definitive guidance on electronic signatures and electronic records has been issued and will support the move to process innovation.<sup>52</sup>

### Harmonization with International Standards (PIC/S)

Blood centers that sell Recovered Plasma in the international market are increasingly subjected to the European interpretation of cGMP. Although there are many similarities between US and European requirements, there are also some striking differences. Awareness of the criteria reflected in the guidance documents issued by the PIC/S will be beneficial in ensuring that inspections by foreign authorities go smoothly.<sup>53-55</sup> Because there have been relatively few inspections carried out by the foreign national authorities, it is not clear how rigidly inspectors will apply the stated requirements summarized below. Different plasma fractionators may have different requirements based on where the end products will be marketed.

### Potential Issues

The QA system must be assessed for effectiveness by management at regular intervals. There must be written delegation of responsibility for hygiene and lot release (lot release requires two senior staff). All records must be kept at least 10 years; donor eligibility and testing records are to be retained at least 30 years (for some fractionators, 33 years). The manual alternatives to automated systems must be exercised with a mock breakdown at least annually. Sterility certificates must be checked before sterile goods are released to inventory. A second person must document performance of a check of all numbers (tubes, product, record forms) after donation. Staff must also control the discard of all unused

numbers. Records storage areas must be locked and have restricted access. Donors may not touch their records except for filling out the initial form. The methods of transport for products and samples must be validated for every phase. Environmental monitoring is required for “clean” work areas. A physician or approved substitute, as well as QA staff, must be present during donor processing.

All manual computer entry must be verified. If samples are pooled for any kind of testing, equivalence study data must be available. Reagent qualification data are required for every lot received. A 2-year retention period is required for test samples. Leukocyte-reduced Red Blood Cells must be shown to have 40 g hemoglobin/unit and hemolysis must be less than 0.8% at the end of shelf life (US products are cleared by the FDA with 1% hemolysis at expiration). Platelets must have a pH of 6.4 to 7.4 at 22°C (vs automated testing at 37°C).

### **Donor Eligibility and Blood Collection**

The eligibility criteria for donors are based upon the Council of Europe “Guide to the Preparation, Use and Quality Assurance of Blood Components.”<sup>56</sup> Some of the differences from the usual US criteria include: donors over 65 years of age need annual physician clearance; males must have a hemoglobin of 13.5 g/dL or higher. Permanent deferral is required for a history of babesiosis, kala-azar, Chagas’ disease, corneal transplant, and convulsions or repeated syncope (or >3 years since last medication without recurrence). Donation is deferred for 6 months (or 4 months with negative NAT) following endoscopy and pregnancy. The donor’s address must be proven and a list of unacceptable addresses posted and periodically updated. The staff must check for a prior deferral and the deferral list details must be defined in writing. The donor’s social security or other identification number is required and donor identity must be confirmed/documented a minimum of twice per visit. The identification step must be repeated before phlebotomy even if the same staff person already identified the donor. Manual blood pressure tests can be performed only by a nurse or physician and all abnormalities are referred to a physician. Tattoos, piercings, branding, or scarification need location and date of procedure noted. Sterility documentation must be from an official source rather than the facility that performed the tattoo or piercing if the donor is accepted with less than a 6-month (for some fractionators, 12-month) wait. Softgoods cannot be set up in advance, including no removal from overwrap until hung. The arm disinfection must be completely dry AND include at least a 30-second wait. It is required to check the product container for defects after collection and to have an independent recheck that all tube, container, and record numbers match. The maximum time allowed between collection and processing must be defined and must be monitored. Complaints require statistical evaluation.

### **Facilities Issues**

The facility must be designed to minimize risk and preclude entry of persons not working in the area. Inspections will assess facility design and size for suitability of rooms and equipment. The collection areas must be suitable for the intended purpose, lack any

incompatible features, and have documentation of QA approval before use. Written assessment of mobile sites before use is required. Testing laboratories should not open to production or storage areas and should preclude donor or public access. Any scissors that are used must be disinfected between cuts of product tubing. Recently, the use of scissors has been banned entirely by some inspectors. Facility design must effectively bar insects and pests of any kind. Cleaning and pest control programs require validation and documentation that includes the products to be used, the areas involved, the methods used, and the schedules established. Segregation of unrelated activities, complete isolation of biohazards, and defined “clean/dirty” pathways are required. No food or drink is allowed in the donor room and all exterior doors are locked if not a defined reception area with continual staff presence. Donor privacy is required for screening and “white noise” is not allowed; there should be no passage way or waiting area next to interview booths. Restrooms should not be available to nondonors. Air flow must be known and be appropriate for clean areas. Walls, floors, and ceilings should all be smooth and cleanable; no carpets or uncleanable mats are permitted. No wooden pallets are permitted. There should be no right of way past biohazardous waste. The biohazardous waste storage area cannot have a re-circulated air supply, and cages are not sufficient isolation. In-use biowaste containers should be visibly clean, be covered or have a restricted opening, and must be emptied daily from the work area. Hand-washing instructions must be posted at each sink.

Freezer validation is critical and must include mapping. Freezing process validation is also required to determine the length of time required to reach a core temperature of –25°C or colder. The freezing process requirements at the time of this writing included that plasma be rapidly frozen at –30°C or colder. Products should be frozen in the coldest area and the alarm and recorder probes should be placed in the warmest spot possible. A difference of at least 2°C (4°C preferred) between the alarm set-point and the maximum freezer temperature is expected. Plasma must be stored and transported at –20°C or colder.

The recorder probe should be in a volume of fluid no larger than smallest product and the fluid should be changed at defined intervals. A back-up thermometer must be inside the freezer and be read daily by staff and documented. Even the defrost cycle cannot exceed the required temperature limit. A freezer map must be posted both inside and out and differentiate where each type of product is stored (untested, tested, quarantine, autologous, released, etc). If a flash freezer is used, staff must document the time required, as determined by study of minimum/maximum loads, etc. Records must include identification of the specific units in each batch as well as the temperature for that batch. Both optical and acoustic alarms are required for freezers. Any removal of product from the freezer must be documented not to exceed a period of 20 minutes at room temperature.

### **Documentation of Look-Back Investigations**

The expected time frames for receipt of viral marker test results must be defined. Notification must be received in less than 7 days

for repeat reactive results and less than 21 days for confirmatory tests. All look-back investigations must be performed within 2 working days. Customer confirmation is required for receipt of notice. Notice must include a period of at least 6 months before the last negative test or 5 years.

## Summary

In this chapter, the development of cGMP elements in the FDA regulation of the pharmaceutical industry and their more extensive application to blood establishments are reviewed. Vigorous application of cGMP principles to the production processes of blood and blood components has greatly reduced, but not completely eliminated, risks associated with the transfusion of blood components. Fortunately, the availability of industrial models broadly applicable to blood centers has facilitated cGMP implementation.

Current good manufacturing practice compliance now represents minimal performance standards for blood centers, and principles of continuous improvement and error rates as low as reasonably achievable have become the hallmarks of quality. Process innovation offers exciting mechanisms for extending the safety of an already exceedingly safe blood supply. Information technology will likely be featured prominently as an increasingly vital part of these innovations.

## Disclaimer

P. A. Hoppe has disclosed a financial relationship with International BioResources, Inc. S. Kochman has disclosed no conflicts of interest.

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**Appendix 64-1.** Top 10 Citations Observed in FDA Inspections Conducted in 2006 (provided by FDA Atlanta District Office)**General Citations**

|                   |                                                                                                                                                                                                                                                                                                                                                                                         |
|-------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 21 CFR 211.22(d)  | The responsibilities and procedures applicable to the quality control unit are not [in writing] [fully followed]. Specifically, ***                                                                                                                                                                                                                                                     |
| 21 CFR 211.100(b) | Written production and process control procedures are not [followed in the execution of production and process control functions] [documented at the time of performance]. Specifically, ***                                                                                                                                                                                            |
| 21 CFR 211.110(a) | Control procedures are not established which [monitor the output] [validate the performance] of those manufacturing processes that may be responsible for causing variability in the characteristics of in-process material and the drug product. Specifically, ***                                                                                                                     |
| 21 CFR 211.100(a) | There are no written procedures for production and process controls designed to ensure that the drug products have the identity, strength, quality, and purity they purport or are represented to possess. Specifically, ***                                                                                                                                                            |
| 21 CFR 211.160(b) | Laboratory controls do not include the establishment of scientifically sound and appropriate [specifications] [standards] [sampling plans] [test procedures] designed to ensure that [components] [drug product containers] [closures] [in-process materials] [labeling] [drug products] conform to appropriate standards of identity, strength, quality, and purity. Specifically, *** |
| 21 CFR 211.165(a) | Testing and release of drug product for distribution do not include appropriate laboratory determination of satisfactory conformance to the [final specifications] [identity and strength of each active ingredient] before release. Specifically, ***                                                                                                                                  |
| 21 CFR 211.192    | There is a failure to thoroughly review [any unexplained discrepancy] [the failure of a batch or any of its components to meet any of its specifications] whether or not the batch has been already distributed. Specifically, ***                                                                                                                                                      |
| 21 CFR 211.188    | Batch production and control records [are not prepared for each batch of drug product produced] [do not include complete information relating to the production and control of each batch]. Specifically, ***                                                                                                                                                                           |
| 21 CFR 211.25(a)  | Employees are not given training in [the particular operations they perform as part of their function] [current good manufacturing practice] [written procedures required by current good manufacturing practice regulations]. Specifically, ***                                                                                                                                        |
| 21 CFR 211.67(b)  | Written procedures are not [established] [followed] for the cleaning and maintenance of equipment, including utensils, used in the manufacture, processing, packing, or holding of a drug product. Specifically, ***                                                                                                                                                                    |

**Device Citations**

|                   |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |
|-------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 21 CFR 820.198(a) | Complaint handling procedures for [receiving] [reviewing] [evaluating] complaints have not been [established] [defined] [documented] [completed] [implemented].                                                                                                                                                                                                                                                                                                                                                                                                                                               |
| 21 CFR 820.100(a) | The procedures for implementing corrective and preventive actions were not [established] [defined] [documented] [complete] [implemented].                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
| 21 CFR 803.17     | Written MDR procedures have not been [developed] [maintained] [implemented].                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
| 21 CFR 820.100(b) | Corrective and preventive action activities have not been documented, including [analysis of sources of quality data] [investigations of causes of nonconformities] [the actions needed to correct or prevent recurrence of nonconforming product and other quality problems] [the verification or validation of corrective actions] [implementation of corrective and preventive actions] [dissemination of information about quality problems or nonconforming product to responsible parties] [submission of information on quality problems and corrective and preventive actions for management review]. |
| 21 CFR 820.75(a)  | A process whose results cannot be fully verified by subsequent inspection and test has not been [adequately] [fully] validated and approved according to established procedures.                                                                                                                                                                                                                                                                                                                                                                                                                              |
| 21 CFR 820.22     | Quality audits were not conducted [at sufficient regular intervals, as prescribed by internal procedures] to verify that the quality system is effective in fulfilling your quality system objectives.                                                                                                                                                                                                                                                                                                                                                                                                        |
| 21 CFR 820.20     | Management with executive responsibility has not ensured that an adequate and effective quality system has been fully implemented and maintained at all levels of the organization.                                                                                                                                                                                                                                                                                                                                                                                                                           |
| 21 CFR 820.22     | Procedures for conducting quality audits were not [established] [defined] [documented] [complete].                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
| 21 CFR 820.30(a)  | Procedures to control the design process of the device were not [established] [defined] [documented] [complete] [implemented].                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| 21 CFR 820.30(i)  | Procedures were not [established] [defined] [documented] [completed] [followed] for the [identification] [documentation] [validation or verification] [review] [approval] of design changes before their implementation.                                                                                                                                                                                                                                                                                                                                                                                      |

**Biologics Citations**

|                   |                                                                                                                                                                                                                                                                                                                                                      |
|-------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 21 CFR 606.100(b) | Written standard operating procedures including all steps to be followed in the [collection] [processing] [compatibility testing] [storage] [distribution] of blood and blood components for [allogeneic transfusion] [autologous transfusion] [further manufacturing purposes] are not always [maintained] [followed] [maintained on the premises]. |
| 21 CFR 606.100(c) | Failure to [perform a thorough investigation] [make a record of the conclusions and follow-up] of [an unexplained discrepancy] [a failure of a lot or unit to meet any of its specifications]. Specifically, ***                                                                                                                                     |
| 21 CFR 606.60(a)  | Equipment used in the [collection] [processing] [compatibility testing] [storage and distribution] of blood and blood components is not [observed] [standardized] [calibrated] on a regularly scheduled basis as prescribed in the SOP manual to ensure that it performs in the manner for which it was designed.                                    |

## Section VI

- 21 CFR 606.160(b) Failure to maintain [donor] [processing] [storage and distribution] [compatibility testing] [quality control] [general] records.
- 21 CFR 606.160(a)(1) Records are not concurrently maintained with the performance of each significant step in the [collection] [processing] [compatibility testing] [storage] [distribution] of each unit of blood and blood components so that all steps can be clearly traced.
- 21 CFR 606.20(b) The personnel responsible for the [collection] [processing] [compatibility testing] [storage] [distribution] of blood or blood components are not adequate in [number] [educational background] [training and experience, including professional training as necessary] to ensure competent performance of their assigned functions, and to ensure that the final product has the safety, purity, potency, identity, and effectiveness it purports or is represented to possess.
- 21 CFR 606.160(a)(1) Records fail to [identify the person performing the work] [include dates of the various entries] [show test results] [include interpretation of the results] [show the expiration date assigned to specific products] [be as detailed as necessary] so as to provide a complete history of the work performed.
- 21 CFR 606.171 Failure to submit a biological product deviation report [within 45 days from the date you acquired information suggesting that a reportable event occurred].
- 21 CFR 606.65(e) Failure to use supplies and reagents in a manner consistent with instructions provided by the manufacturer.

### Drug Citations

- 21 CFR 211.22(d) The responsibilities and procedures applicable to the quality control unit are not [in writing] [fully followed].
- 21 CFR 211.100(b) Written production and process control procedures are not [followed in the execution of production and process control functions] [documented at the time of performance].
- 21 CFR 211.110(a) Control procedures are not established which [monitor the output] [validate the performance] of those manufacturing processes that may be responsible for causing variability in the characteristics of in-process material and the drug product.
- 21 CFR 211.160(b) Laboratory controls do not include the establishment of scientifically sound and appropriate [specifications] [standards] [sampling plans] [test procedures] designed to ensure that [components] [drug product containers] [closures] [in-process materials] [labeling] [drug products] conform to appropriate standards of identity, strength, quality, and purity.
- 21 CFR 211.100(a) There are no written procedures for production and process controls designed to assure that the drug products have the identity, strength, quality, and purity they purport or are represented to possess.
- 21 CFR 211.165(a) Testing and release of drug product for distribution do not include appropriate laboratory determination of satisfactory conformance to the [final specifications] [identity and strength of each active ingredient] before release.
- 21 CFR 211.192 There is a failure to thoroughly review [any unexplained discrepancy] [the failure of a batch or any of its components to meet any of its specifications] whether or not the batch has been already distributed.
- 21 CFR 211.25(a) Employees are not given training in [the particular operations they perform as part of their function] [current good manufacturing practice] [written procedures required by current good manufacturing practice regulations].
- 21 CFR 211.188 Batch production and control records [are not prepared for each batch of drug product produced] [do not include complete information relating to the production and control of each batch].
- 21 CFR 211.67(b) Written procedures are not [established] [followed] for the cleaning and maintenance of equipment, including utensils, used in the manufacture, processing, packing or holding of a drug product.

### Bioresearch Monitoring Citations

- 21 CFR 312.60 An investigation was not conducted in accordance with the [signed statement of investigator] [investigational plan]. Specifically\*\*\*
- 21 CFR 312.62(b) Failure to prepare or maintain [adequate] [accurate] case histories with respect to [observations and data pertinent to the investigation] [informed consent].
- 21 CFR 312.62(a) Investigational drug disposition records are not adequate with respect to [dates] [quantity] [use by subjects].
- 21 CFR 56.115(a)(2) Minutes of IRB meetings have not been [prepared] [maintained] in sufficient detail to show [attendance at the meetings] [actions taken by the IRB] [the vote on actions, including the number of members voting for, against and abstaining] [the basis for requiring changes in or disapproving research] [a written summary of the discussion of controverted issues and their resolution].
- 21 CFR 312.60 Failure to obtain informed consent in accordance with 21 CFR Part 50 from each human subject before [drug administration] [conducting study-related tests] . Specifically\*\*\*
- 21 CFR 56.108(a)(1) The IRB [has no] [did not follow its] written procedure for conducting its [initial] [continuing] review of research.
- 21 CFR 312.66 Failure to report promptly to the IRB all unanticipated problems involving risk to human subjects or others.
- 21 CFR 50.27(a) Informed consent was not properly documented in that the written informed consent used in the study [was not approved by the IRB] [was not signed by the subject or the subject's legally authorized representative at the time of consent ] [was not dated by the subject or the subject's legally authorized representative at the time of consent].
- 21 CFR 56.108(c) For other than expedited reviews, the IRB does not always review proposed research at convened meetings at which a majority of the members of the IRB are present, including at least one member whose primary concerns are in nonscientific areas.

- 21 CFR 56.115(a)(5) A list of IRB members has not been [prepared] [maintained], identifying members by [name] [earned degrees] [representative capacity] [indications of experience sufficient to describe each member's chief anticipated contribution to IRB deliberations] [any employment or other relationship between each member and the institution].

#### HCT/P Citations

- 21 CFR 1271.47(a) Procedures for all steps performed in the [testing] [screening] [determining] of donor eligibility of HCT/Ps were not [established] [maintained] [defined] [documented] [implemented] [followed] [reviewed] [revised].
- 21 CFR 1271.180(a) Procedures appropriate to meet core cGTP requirements for all steps that you perform in the manufacture of HCT/Ps were not [established] [maintained] [defined] [documented] [implemented] [followed] [reviewed] [revised].
- 21 CFR 1270.31(d) Failure to [prepare] [validate] [follow] written procedures for prevention of [infectious disease contamination] [cross-contamination] during processing.
- 21 CFR 1270.31(b) Failure to [prepare] [follow] written procedures for all significant steps for [obtaining] [reviewing] [assessing] the relevant medical records of a donor.
- 21 CFR 1271.75(a)(1) Donors were not screened by a review of relevant medical records for [risk factors] [clinical evidence] of communicable disease agents and diseases.
- 21 CFR 1270.33(a) Failure to maintain records which are [accurate] [indelible] [legible].
- 21 CFR 1271.200(b) Procedures for the [cleaning] [sanitizing] [maintenance] of equipment were not [established] [maintained] [defined] [documented] [implemented] [followed] [revised].
- 21 CFR 1271.160(c) Periodic quality audits of activities related to core cGTP requirements have not been performed.
- 21 CFR 1270.33(a) Records fail to [identify the person performing the work] [include the dates of the various entries] [be as detailed as necessary to provide a complete history of the work performed and to relate the records to the particular tissue involved].
- 21 CFR 1271.160(b)(1) The quality program has not ensured that appropriate procedures related to core cGTP requirements were [established] [maintained] [defined] [documented] [implemented] [followed] [reviewed] [approved] [revised].

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MDR = medical device report; SOP = standard operating procedure; IRB = institutional review board; HCT/P = human cells, tissues, and cellular and tissue-based products; cGTP = current good tissue practice.



# Transplant Organizations and Networks in the Regulation of Cellular Therapy Programs

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Worldwide, multiple governmental and nongovernmental organizations are involved in various aspects of cellular therapy. Understanding these organizations and their roles provides useful insights on the current and future directions in this field. Practically speaking, most of the existing structure has developed to support only one cellular therapy—namely, hematopoietic stem/progenitor cell transplantation (HSCT). As additional cellular therapies emerge, new entities might develop in support of these therapies or the existing organizations might enlarge their scope to embrace new aspects of the field. This type of evolution is particularly well illustrated by the development in recent years of umbilical cord blood (UCB) as a useful graft source for allogeneic HSCT. Several organizations with long-standing roles in traditional HSCT have expanded their activities to include UCB transplantation. At the same time, new organizations, eg, NetCord and Eurocord, have also emerged to play important roles in this developing field.

## Hematopoietic Stem/Progenitor Cell Transplantation

The therapeutic use of HSCT has been expanding for nearly 40 years.<sup>1</sup> Today, an estimated 60,000 HSCT procedures are performed annually, worldwide. Twenty-thousand of these are allogeneic transplants in which the stem cell graft has been procured from a living donor or from the placental/umbilical cord blood of a newborn. Marrow collected under general anesthesia from living donors was previously the sole graft source for HSCT, but today peripheral blood progenitor cells (PBPCs) are employed for virtually all autologous HSCTs and for more than 70% of allogeneic living donor HSCTs. The use of UCB units for HSCT continues to be more prevalent among recipients who are children or adolescents, where UCB units are used in 20% to 25% of allogeneic HSCTs.

The most common indications for allogeneic transplantation are hematologic malignancies, eg, acute myelogenous leukemia, acute lymphocytic leukemia, and myelodysplastic syndrome. Chronic myelogenous leukemia was previously the single most common allogeneic HSCT diagnosis, but transplants for this condition have declined with the development of tyrosine kinase inhibitors, especially imatinib.<sup>2-4</sup> Severe aplastic anemia and other marrow failure states are the most common nonmalignant conditions that are indications for allogeneic HSCT. Up to 40% of transplant procedures for children, however, are performed for nonmalignant conditions including congenital metabolic disorders and inherited and acquired immune system abnormalities.

HLA matching between the donor and recipient plays a critical role in the success of allogeneic HSCT.<sup>5-10</sup> The HLA Class I proteins, HLA-A, -B, and -C, are expressed on the surfaces of most cells in the human body. The Class II proteins, HLA-DR, -DQ, and -DP, are more restricted in their natural expression, but can be induced by various stimuli. HLA is fully discussed in Chapter 57.

The HLA proteins are encoded by genes that reside in a relatively small region on the short arm of chromosome 6. Because they are close together, the group of genes, termed a haplotype, tends to be inherited en bloc. Thus, in the absence of chromosome crossovers in the HLA region, which occur at a frequency of approximately 5%, all the offspring of a particular couple will demonstrate one of four HLA phenotypes. Therefore, each full sibling of an individual in need of allogeneic HSCT has a 25% chance of being an HLA match. There is a 50% chance the sibling, like the parents, will be half matched (haploidentical) and a 25% chance the sibling will share no HLA haplotypes with the HSCT candidate. In the United States, Canada, and Europe, family sizes are such (average of just over two children) that if one family member is an HSCT candidate, the likelihood of an HLA-matched sibling donor is about 30%. This fact alone has driven the worldwide development of large registries composed of HLA-typed adult volunteers who are willing to consider anonymous HSCT donation. The more recent development of allogeneic UCB banking also stems from the recognition that most

individuals who are candidates for HSCT lack suitably matched family-member donors.

## Donor Registries and Their Networks

### National Marrow Donor Program

The National Marrow Donor Program (NMDP) was established in 1986 to recruit adults between the ages of 18 and 60 to serve as marrow donors for unrelated HSCT.<sup>11-13</sup> Since that time, NMDP has grown to become the world's largest unrelated donor registry. The current NMDP file lists more than 7.1 million adult donors and 82,000 UCB units (Table 65-1). Twenty-one percent of the adult donors have HLA typing only at HLA-A and -B loci. If there are no suitable HLA-A, -B, and -DRB1 donors present on a patient search, selective DRB1 typing of matched HLA-A, -B-only donors may uncover a complete match. In practice this is uncommon because the HLA-A, -B-only pool of donors is composed largely of HLA-A, -B phenotypes that are common in those of European ancestry. In previous years, NMDP developed prospective DRB1 typing programs to scour the pool of HLA-A, -B-only donors and complete the typing on those likely to represent unique HLA phenotypes. All newly recruited donors and UCB units are HLA-typed at HLA-A, -B, and -DRB1 loci by highly accurate DNA-based methods.

NMDP has developed and maintains standards for participation in and operation of its network.<sup>14</sup> The network includes 171 transplant centers, which are HSCT programs within large tertiary care hospitals, many of them university medical centers. Adult donors are recruited and managed by one of 70 member donor centers. These donor centers are based at community blood centers, universities, or freestanding facilities. Donor centers are assisted in their recruitment activity by 11 recruitment groups, which are organizations focused upon recruitment within US racial and ethnic minority populations. When a donor is selected to provide PBPCs for transplantation, the collection occurs at one of 91 designated apheresis centers. Similarly, marrow is collected at one of 97 hospitals, which are designated as member collection centers. UCB units, the newest graft source for unrelated donor transplantation, are

collected and stored at 24 member cord blood banks. Many of the centers within the NMDP network are located outside the United States, including 43 transplant centers, six donor centers, nine apheresis centers, 16 collection centers, and three cord blood banks. Additional adult donors and UCB units can be accessed through formal agreements with 26 international cooperative registries.

The NMDP recruits about 350,000 new US adult donors annually. Almost all of these are recruited at community donor drives, which are held at numerous locations including shopping malls, blood centers, college campuses, community centers, and churches. More than 1000 drives are held each month. Prospective recruits are screened to confirm good health and the absence of high-risk behaviors,<sup>15</sup> after which informed consent is obtained. Blood samples for HLA typing have been largely replaced by swabs of the buccal mucosa. In the early years of the NMDP registry, donors were HLA typed by serologic methods, but these have been replaced by highly accurate DNA-based HLA typing methods. However, because of cost considerations, donors are not typed at the highest available resolution (allele-level), but are instead typed at an intermediate level of resolution. Once HLA typing is completed, the results, along with limited demographic information and the assigned donor identification number, are entered into the NMDP databases where they become available to searching patients.

The processes in place at cord blood banks are similar.<sup>16-20</sup> Each bank may work with one or more hospital-based obstetric programs. Expectant mothers are recruited through the obstetric program or through doctors' offices. Mothers complete a health questionnaire and provide consent for unit collection before delivery. UCB units that are deemed suitable undergo processing, testing, and cryopreservation at the cord blood bank. When the necessary criteria are met, the units are listed in the registry and made available for searching. Further details about umbilical cord blood are presented in Chapter 36.

Transplant centers manage searches for their patients who are prospective HSCT candidates. Several studies have shown that HLA matching is critical for the success of unrelated allogeneic transplantation. With adult donors, patient outcomes are best when the donor and recipient are allele-level matched at HLA-A, -B, -C, and -DRB1 loci.<sup>5-7,21,22</sup> UCB unit transplantation does not appear to require the same high level of HLA matching.<sup>23-26</sup> UCB units are typically matched by intermediate resolution at HLA-A and -B loci, and high resolution at HLA-DRB1 loci. UCB units should be matched to at least the 4 of 6 level, but 5 of 6 or 6 of 6 is preferred. Cell dose also plays a critical role in the success of UCB HSCT, so unit selection must consider both HLA match and cell dose.<sup>26-28</sup>

Internet-based computer software enables transplant centers to consider these complex factors and examine the NMDP registry of adult donors and UCB units. Because adult donors and UCB units are not HLA-typed at high resolution, a sophisticated computer algorithm, designated HapLogic, has been developed

**Table 65-1.** Composition of the NMDP Donor and Cord Blood Unit File (as of June 2008)

|                                | Total     | HLA-A, -B and -DR<br>Typed (% of Total) |
|--------------------------------|-----------|-----------------------------------------|
| NMDP US adult donors           | 4,869,783 | 4,148,350 (85)                          |
| NMDP international<br>donors   | 2,040,444 | 1,332,664 (65)                          |
| Cooperative registry<br>donors | 193,040   | 138,434 (72)                            |
| Total adult donors             | 7,103,267 | 5,619,448 (79)                          |
| UCB units                      | 82,562    | 82,562 (100)                            |

NMDP = National Marrow Donor Program; UCB = umbilical cord blood.

to predict the likelihood that any given donor or UCB unit is allele-level-matched with the prospective recipient. HapLogic narrows the donor selection process so the transplant center can select a few adult donors or UCB units for further evaluation, including high-resolution HLA typing. This stage of the search process is termed “confirmatory testing.” After confirmatory testing, a final donor selection is made. If a UCB unit is selected, the cord blood bank will transport the unit frozen in liquid nitrogen to the transplant center. If an adult donor is selected, the donor will be scheduled for a marrow or PBPC collection at a collection center or apheresis center, respectively, preferably near the donor’s residence. Fresh marrow and PBPC products are transported by trained couriers to the designated transplant center where the product is promptly administered to the waiting HSCT recipient.

Other registries and cord blood banking operations are organized similarly to the NMDP network. There are far too many such entities to discuss in detail, but several are listed in Table 65-2 and a few are specifically discussed below.

**Caitlin Raymond International Registry and Gift of Life Bone Marrow Registry**

Caitlin Raymond International Registry (CRIR) and Gift of Life Registry are two registries with headquarters in the United States. CRIR was founded in 1986 and has its offices at the University of Massachusetts Memorial Medical Center, Worcester, MA. The registry is named after the founder’s daughter, who had developed juvenile myelomonocytic leukemia and received a transplant from an unrelated donor. Approximately 75,000 adult donors are registered with CRIR. Gift of Life was founded 1991 to recruit primarily donors of Jewish ethnicity. The founder and current executive director was himself a leukemia patient in need of a transplant donor. He received his transplant in 1995 and has

continued to operate Gift of Life to benefit other patients. Gift of Life has recruited about 122,000 adult donors and has also recently established a UCB bank. Donors from both CRIR and Gift of Life are listed in the NMDP file as cooperative registry donors and are directly accessible.

**Anthony Nolan Bone Marrow Trust**

The Anthony Nolan Bone Marrow Trust (ANBMT) was established in London in 1974. Like the Caitlin Raymond Registry, this registry was named for a child in need of an unrelated donor transplant. Today the ANBMT maintains a file of more than 390,000 donors and has facilitated more than 8000 transplant procedures. In addition, ANBMT supports an extensive research program focused on the immunobiology of unrelated donor HSCT. Additional registries in the United Kingdom and Ireland include the British Bone Marrow Registry, the Welsh Bone Marrow Donor Registry, and the Irish Unrelated Bone Marrow Registry.

**German National Bone Marrow Donor Registry**

The most active registry in the world is the German National Bone Marrow Donor Registry [Zentrale Knochenmarkspender-Register für die Bundesrepublik Deutschland (ZKRD)]. ZKRD has more than 3.2 million adult donors distributed among 31 donor centers and has collected 6500 UCB units. In 2006, ZKRD provided 3058 adult donor and cord blood products for transplantation compared to 2681 products released by the NMDP. More than 60% of the products released by ZKRD in 2006 were exported to other nations, mostly in the European Union. The products not exported were distributed to 33 transplant centers located within Germany. The vast majority (83%) of adult donor products collected by ZKRD in 2006 were PBPCs (2491 PBPC units vs 548 marrow).

**Table 65-2.** Selected Registries of Adult Donors and Umbilical Cord Blood\*

| Name                                             | Country   | Adult Donors | Cord Blood Units |
|--------------------------------------------------|-----------|--------------|------------------|
| Caitlin Raymond International Registry           | US        | 75,484       | 11,030           |
| Gift of Life Bone Marrow Registry                | US        | 122,422      | 927              |
| Australia Bone Marrow Donor Registry             | Australia | 159,694      | †                |
| Canadian Unrelated Bone Marrow Donor Registry    | Canada    | 229,989      | NL‡              |
| Chinese Marrow Donor Program                     | China     | 554,495      | NL               |
| France Greff de Moelle                           | Franca    | 157,269      | †                |
| ZKRD, German National Bone Marrow Donor Registry | Germany   | 3,214,716    | 5,383            |
| Ezer Mizion Bone Marrow Donor Registry           | Israel    | 355,144      | NL               |
| Italian Bone Marrow Donor Registry               | Italy     | 324,773      | †                |
| Japan Marrow Donor Program                       | Japan     | 307,786      | NL               |
| Buddhist Tzu Chi Stem Cells Center               | Taiwan    | 275,378      | 4,426            |
| Anthony Nolan Bone Marrow Trust                  | UK        | 390,788      | NL               |
| NetCord                                          | Multiple  | NL           | 153,830§         |

\* Except as noted, adult donor and cord blood unit inventories are from Bone Marrow Donors Worldwide (<http://www.bmdw.org>) as of June 2008.

†Cord Blood units are included in the NetCord inventory.

‡NL: Not listed with the registry as of 2006.

§As of October 2007.

## NetCord

NetCord was established in 1999 as an international organization of cord blood banks. A total of 25 cord blood banks are members of NetCord with a combined inventory in excess of 150,000 UCB units. As of October, 2007, NetCord banks had provided 6227 UCB units for transplantation. The majority of these units were used for children, but UCB unit transplantation for adults is continuing to increase. Since 2000, NetCord has partnered with the Foundation for the Accreditation of Cellular Therapy (FACT) to develop international standards for cord blood unit collection, processing, testing, storage, and release. The NetCord-FACT standards are currently in their third edition. NetCord and NMDP have also recently entered into a collaboration that allows the exchange of search information.

## World Marrow Donor Association

The World Marrow Donor Association (WMDA) is an organization of international registries and cord blood banks working together to promote international cooperation and the exchange of HSCT products.<sup>29,30</sup> WMDA was established in 1994 and maintains several working groups that address issues in quality assurance, registry operations, ethics, finances, and transplant program services.<sup>29-34</sup> WMDA has also established standards for the operation of unrelated donor registries and created an accreditation program for its members.<sup>35</sup> In 1999 WMDA added cord blood banks to its membership, which currently totals 67 adult donor registries and 48 cord blood banks.

The WMDA conducts an annual survey of its members. Sixty-seven registries and 47 cord blood banks responded to the most recent survey, which covered activities through the end of 2006.<sup>36</sup> The survey documented more than 11,800,000 potential adult HSCT donors and 292,000 UCB units worldwide. Virtually all of the UCB units and about 70% of the adult donors had been tissue typed for HLA-A, -B, and -DRB1.

Adult donor registries collected 8418 unrelated donor products in 2006. PBPC products dominated with 5416 (64% of total) compared to marrow (3002 products, 36% of total). Among the adult donor products collected, 7975 (95%) were first-time donations, but 443 represented products from donors who had previously donated for the same or a different HSCT recipient. The 47 cord blood banks in the survey reported releasing 2075 UCB units for transplantation in 2006. The exact number of UCB recipients is uncertain because of the popular rise in the frequency of double-unit UCB transplantation.<sup>37</sup>

It is important to emphasize the prominent international nature of unrelated donor HSCT activities. In 2006, 3269 (39%) adult products and 829 (40%) UCB units were shipped to transplant centers in other countries. This robust international activity is apparently driven by the need for HLA tissue matching and facilitated by electronic search engines, such as that of the NMDP, which provide rapid access to international inventories of adult donors and UCB units.

A very useful tool for initiating international searches is offered by Bone Marrow Donors Worldwide (BMDW).<sup>38</sup> Most WMDA

members list donor information in this database where it is accessible through the Internet. A BMDW search will quickly direct transplant teams to those registries and cord blood banks that are most likely to have suitable HLA matches. As of mid-2008, 12.2 million adult donors and 299,000 UCB units were listed in BMDW from 97 adult donor and cord blood programs in 45 countries.

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## Outcomes Registries, Professional Associations, and Their Networks

### Center for International Blood and Marrow Transplant Research

The Center for International Blood and Marrow Transplant Research (CIBMTR) was established in 2004 through an affiliation of the International Bone Marrow Transplant Registry (IBMTR) and research programs of the NMDP. The affiliation brought together two organizations with a long histories and extensive experience in collection of data concerning the results of HSCT.<sup>12,39</sup> The IBMTR was established in 1972, shortly after the first successful allogeneic HSCT experiences were reported. The goal of IBMTR was to collect data about transplantation that could be used to analyze trends, evaluate efficacy, and conduct research concerning HSCT. IBMTR created an open structure of working committees focused on particular diseases and key topics affecting HSCT. The emergence of autologous HSCT led IBMTR to establish the companion Autologous Blood and Marrow Transplant Registry (ABMTR) with additional working committees. Membership in IBMTR/ABMTR and the submission of transplant outcome data were voluntary activities for transplant programs. In contrast, the submission of outcome data to NMDP for unrelated donor transplant recipients was a requirement of congressional legislation. Both organizations maintained computer databases, staff for auditing and data management, and research agendas. The complementary strengths of IBMTR and NMDP and the desire to reduce duplication of effort led to the merger of research activities and creation of CIBMTR.

Currently CIBMTR has participation from more than 400 transplant programs in 47 countries. Data have been accumulated on nearly one-quarter million autologous and allogeneic transplant procedures. CIBMTR has developed two levels of data reporting for its centers. The first level collects basic information about an HSCT procedure and its outcome. This is termed "transplant essential data" (TED) and is useful for monitoring trends and conducting basic analyses. The second level of data collection involves comprehensive, research-level data. These data are collected on selected patients based on diagnosis, type of donor, type of transplant procedure, and other factors.

The research structure of CIBMTR includes 17 scientific working committees, which receive, review, and prioritize research studies. A steering committee also oversees access to research blood samples from HSCT donors and recipients maintained in a repository operated by NMDP. In addition, CIBMTR has established another new program (Resource for Clinical Investigation in BMT), which is designed to facilitate multicenter, prospective

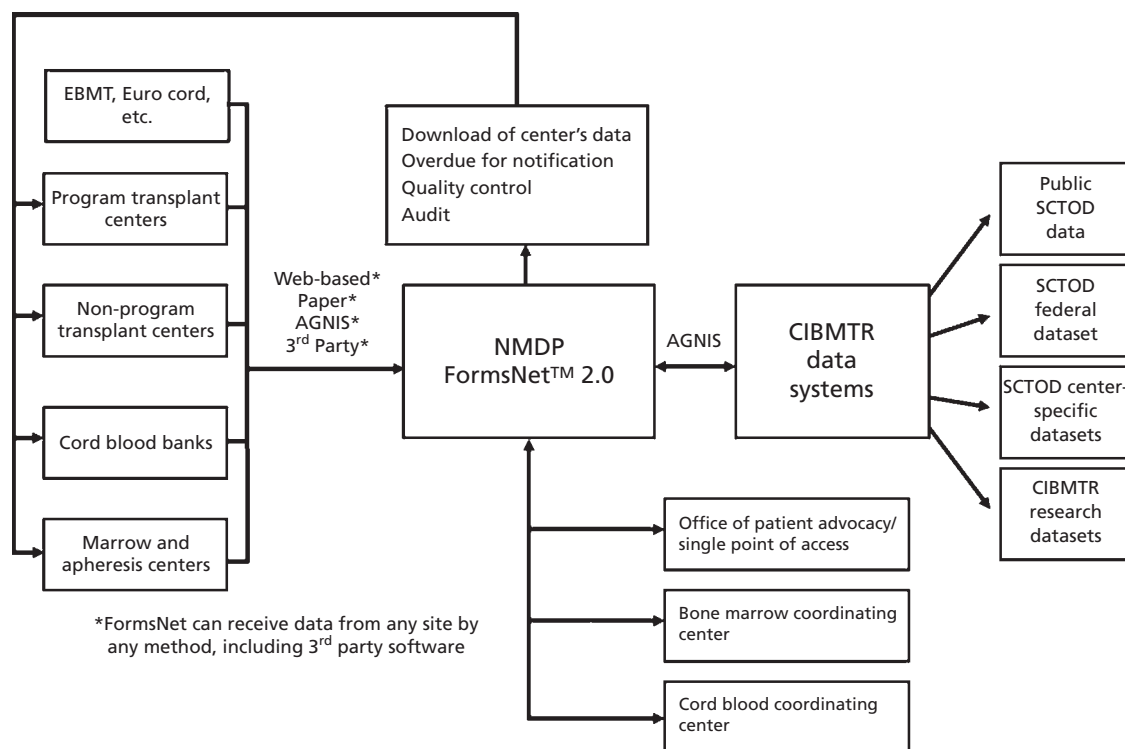


**Figure 65-1.** Data management system of the CIBMTR. Data are submitted by participating organizations to NMDP FormsNet 2.0. FormsNet communicates with the organizations submitting data, with components of the NMDP programs, and with the CIBMTR data systems. CIBMTR distributes SCTOD data and research datasets. AGNIS: A Growable Network Information System.

clinical trials. The annual research agenda of CIBMTR is largely established at its annual meeting, the BMT Tandem Meetings, held in partnership with the American Society for Blood and Marrow Transplantation (ASBMT) in February of each year.

In 2006, the CIBMTR was awarded a contract from the US government to develop and operate the Stem Cell Therapeutic Outcomes Database (SCTOD). The Stem Cell Therapeutic and Research Act of 2005 created the SCTOD with a mandate to collect HSCT outcomes data from every allogeneic HSCT procedure that involves a US recipient or donor.<sup>40</sup> The Act required, among other things, that data from the SCTOD would be used to evaluate the performance of individual transplant centers and that portions of the data would be made publicly available. In fulfillment of the SCTOD requirements, CIBMTR expanded the TED forms to collect sufficiently detailed data for the required analyses. Expansion of TED was pursued in collaboration with the European Group for Blood and Marrow Transplantation (EBMT) and the Asia-Pacific Blood and Marrow Transplant Group (APBMT), so that a new international standard for the minimal acceptable HSCT dataset was established. Subsequently, CIBMTR with support from the NMDP launched a new data collection and management program in December 2007 (Fig 65-1). The new program, which collects both SCTOD data (TED data) and comprehensive research-level data, employs a web-based data entry and validation system, FormsNet 2.0, as well as an emerging component, AGNIS, for automated messaging of data. AGNIS, which is the acronym

for A Growable Network Information System, was started with funding from the National Institutes of Health Roadmap Initiative and relies heavily on tools developed by the National Cancer Institute's cancer Biomedical Informatics Grid (caBIG) project. AGNIS seeks to reduce the data management burden for transplant teams by establishing a system of automated, authenticated, and secure transmission of HSCT outcomes data among designated, participating databases. To establish the semantic connection for AGNIS data, the acceptable common data elements are fully defined in the publicly available cancer Data Standards Repository (caDSR) managed by the NCI. AGNIS is open source software available for download (<http://www.agnis.net>).

### American Society for Blood and Marrow Transplantation

The ASBMT is a professional society whose members include transplant physicians and allied health-care personnel. ASBMT promotes the clinical and research interests of the HSCT community with its membership, the pharmaceutical industry, and national government. Through these efforts, ASBMT addresses research initiatives and funding, communication and education about HSCT activities, establishment of clinical standards, and reimbursement by payers. ASBMT publishes a peer-reviewed journal, *Biology of Blood and Marrow Transplantation*. Its annual meeting, the BMT Tandem Meetings, is held in partnership with the CIBMTR. Together with the International Society for Cellular Therapy, ASBMT established FACT.

## AABB

AABB was established in 1947 as professional organization in transfusion medicine. Its tag line—Advancing Transfusion and Cellular Therapies Worldwide—was adopted in recognition of its international presence and expanding role in cellular therapies beyond traditional blood transfusion. More than 2000 institutions and 8000 individuals are members of AABB. The organization sets standards and operates an accreditation program for transfusion services and cellular therapy processing programs. The *AABB Standards for Cellular Therapy Product Services* is currently in its 3rd edition.<sup>41</sup> AABB programs and services address regulatory and legal matters, promote education and communication, and publish electronic and print resources. AABB publishes a monthly peer-reviewed journal, *Transfusion*, and holds its annual meeting in the fall of each year.

## European Group for Blood and Marrow Transplantation

The EBMT functions as both a professional organization and as an HSCT outcomes registry. The organization combines features of both CIBMTR and ASBMT. EBMT was founded in 1974 to allow scientists and physicians involved in clinical HSCT to share their experiences and engage in collaborative studies. The official journal of EBMT is *Bone Marrow Transplantation*.

More than 500 organizations report outcomes data to EBMT. Through continuing harmonization efforts with CIBMTR, the EBMT minimal clinical dataset (MED A) is identical to the CIBMTR TED. Like CIBMTR, EBMT also collects a comprehensive research dataset (MED B). EBMT holds its annual meeting in the spring of each year.

## Eurocord

Eurocord, like NetCord, emerged to fill a niche created by the appearance of cord blood as a new and promising HSCT cellular therapy. It was established in 1995 on behalf of EBMT in order to promote and evaluate umbilical cord blood transplantation. Eurocord collects transplant outcome data from EBMT and non-EBMT transplant centers and merges these data with UCB unit data provided by cord blood banks. More than 3300 UCB HSCT cases have been reported to Eurocord. Eurocord shares UCB data with EBMT and collaborates closely with NetCord and CIBMTR in sharing data and conducting cord blood transplantation research.

## Asia-Pacific Blood and Marrow Transplantation Group

The APBMT is an association of 12 Asian countries organized for the purpose of encouraging and enhancing HSCT activity throughout Asia. In 2006, at the 11th Annual Congress of APBMT in Nagoya, Japan, participants agreed to establish the Asian BMT Registry for the collection of HSCT outcomes data. APBMT subsequently adopted the TED/Med A dataset of CIBMTR/EBMT. In 2007, APBMT conducted a survey of HSCT activity in which seven of its member nations participated.<sup>42</sup> The survey documented 4500 total transplants in 2005, of which two-thirds were allogeneic transplants. Japan accounted for most (75%) of the transplant activity, but China exhibited the most rapidly increasing transplant numbers.

## International Society for Cellular Therapy

The International Society for Cellular Therapy (ISCT) is a professional organization established in 1992 for individuals involved in cell-based research, processing, manipulation, and clinical therapy. The society pursues scientific and educational activities in cellular therapy and seeks to influence regulatory authorities in the North America, Europe, and elsewhere. ISCT holds its annual meeting in the spring of each year. The Society's journal is *Cytotherapy*. FACT was cofounded by ISCT and ASBMT.

ISCT Europe is a regional branch of the organization with its own leadership. ISCT Europe partnered with EBMT in the founding of the Joint Accreditation Committee—ISCT and EBMT (JACIE).

## Worldwide Group for Blood and Marrow Transplantation

The Worldwide Group for Blood and Marrow Transplantation (WBMT) is a recently established collaboration created with the goal of encouraging, harmonizing, and compiling international HSCT outcomes data. Founding partners in WBMT include CIBMTR, EBMT, APBMT, and WMDA. Just as WMDA exists as a collaboration of international HSCT graft suppliers, WBMT seeks to stimulate and enhance international collection of standardized HSCT outcomes data. WBMT is early in its organizational history, but activities on its preliminary docket include: 1) maintaining consensus on a standard outcomes dataset for HSCT recipients and donors, 2) encouraging the development and participation of regional outcomes registries, 3) establishing a nomenclature system for unambiguous identification of centers engaged in HSCT activities, 4) establishing systems for unambiguous identification of recipients and donors that also preserve confidentiality and privacy, and 5) investigating the practicality and utility of periodic global surveys of HSCT activity.

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## Accrediting Organizations

Official governmental agencies (also called the competent authorities) regulate cellular therapies. The transplant community's achievement of regulatory compliance, however, is in large part buttressed by voluntary accrediting organizations, established by professional associations in order to provide self-assessment and self-regulation. As previously discussed, WMDA has established a voluntary accreditation program for international unrelated donor registries. This program is being phased-in over several years. Currently 12 WMDA members have received accreditation certification.

## AABB

AABB has a long history of standard-setting and accreditation for blood services. In the last 10 years, AABB accreditation has been extended to facilities engaged in the collection, processing, and release of cellular therapy products. The *AABB Standards for Cellular Therapy Product Services* covers marrow, PBPC, and UCB collections.<sup>41</sup> AABB-accredited laboratory and cell processing-based facilities include 43 cord blood banks, 106 cell therapy programs, and two somatic cell services in 16 countries.

### Foundation for the Accreditation of Cellular Therapy

FACT was co-founded by ASBMT and ISCT in 1996.<sup>43,44</sup> FACT accredits both laboratory-based programs and clinical programs engaged in cellular therapies. In contrast to AABB, FACT has established a separate set of standards for cord blood banks in collaboration with NetCord. The *NetCord-FACT International Standards for Cord Blood Collection, Processing, Testing, Banking, Selection and Release* address the stated components of cord blood banking.<sup>45</sup> The standards for clinical HSCT programs, as well as collection, processing, and distribution of cells isolated from marrow and blood, are addressed in the *FACT-JACIE International Standards for Cellular Product Collection, Processing and Administration*.<sup>46</sup> As implied by the title, these standards have been developed in collaboration with JACIE.

FACT has accredited 15 cord blood banks in nine countries. Accreditation under FACT-JACIE standards is complex because a center may be accredited for any combination of seven categories, which include cell processing, marrow collection, PBPC collection, adult allogeneic HSCT, adult autologous HSCT, pediatric allogeneic HSCT, or pediatric autologous HSCT. FACT has accredited cell processing in 156 programs in the United States and Canada. FACT accredits 150 programs for hematopoietic cell collections and processing and 140 for HSCT.

### Joint Accreditation Committee—ISCT and EBMT

JACIE was established in 1998.<sup>47,48</sup> As noted above, it accredits according to the harmonized *FACT-JACIE International Standards for Cellular Product Collection, Processing and Administration*.<sup>46</sup> JACIE has accredited 52 programs in 10 European Union nations. Thirty-nine programs are accredited for cell processing, 46 for hematopoietic cell collections, and 41 for HSCT.

### Alliance for Harmonisation of Cellular Therapy Accreditation

The Alliance for Harmonisation of Cellular Therapy Accreditation (AHCTA) was established in 2006 with the intent to provide international harmonization of the cellular therapy standards and to represent the standard-setting organizations to the various regulatory authorities worldwide. Current members of AHCTA include AABB, ASBMT, EBMT, the European Foundation for Immunogenetics, FACT, ISCT, ISCT-Europe, JACIE, the NetCord Foundation, and WMDA. AHCTA has created a position paper on global standards in cellular therapies and is working toward a detailed crosswalk of various regulations and professional standards in existence today.

## Governmental Agencies in Cellular Therapy

### Health Resources and Services Administration

Governmental agencies are involved in diverse aspects of cellular therapies. Governmental agencies also provide funding for the conduct of laboratory and clinical research in support of cellular therapies. In the United States, the National Institutes of Health is the primary health research funding agency. However,

governmental funding also provides programmatic support for enhancing cellular therapies. In 2005, Congress passed the Stem Cell Therapeutic and Research Act.<sup>40</sup> The Act established two components for the advancement of cellular therapies. The first, the C. W. Bill Young Cell Transplantation Program (the Program) was created to extend the work of the previous congressionally authorized National Bone Marrow Donor Registry. The Program recognizes Congressman C. W. Bill Young (R) from Florida, who has been a long-time supporter of federal funding for the development of unrelated donor registries and the expansion of HSCT therapies. The second component of the Act established the National Cord Blood Inventory (NCBI) with a goal to collect and store 150,000 high-quality UCB units in public banks. The Act contained other noteworthy features. It required that one or more organizations would be selected for accrediting the cord blood banks participating in the NCBI program. Further, it established an Advisory Council on Blood Stem Cell Transplantation, that reports to the Secretary of Health and Human Services (HHS) with advice on the matters related to the Program and the NCBI. Responsibility for implementing the requirements of the Act fell to the Health Resources and Services Administration (HRSA) within HHS. HRSA established four contracts for operation of the Program and through a competitive bidding process these were awarded to NMDP and CIBMTR. In addition HRSA has awarded NCBI contracts to eight cord blood banks.

Program and NCBI information for potential transplant recipients, their families, potential donors, and the public is provided online (<http://bloodcell.transplant.hrsa.gov>). Twenty years of federal funding for the establishment of adult donor registries and more recently umbilical cord blood registries in the United States have had a major impact. Much of the progress in unrelated donor HSCT has accrued because of congressional leadership, and thoughtful implementation by federal agencies.

### World Health Organization

Another type of governmental programmatic support is provided by the World Health Organization (WHO). The transplantation program of WHO has held two global consultations on the subject of human cells and tissues for transplantation. These consultations addressed the global needs for systems of vigilance and surveillance to ensure the safety of cell and tissue transplantation. The consultations led to the development of two Aide-Memoire documents, which establish basic requirements in the field of cell and tissue transplantation.<sup>49,50</sup> In addition, the WHO transplantation program has established the Global Knowledge Base on Transplantation (GKT), which is envisioned as a database of information on transplantation of organs, cells, and tissues from around the world. The GKT is intended to address four topics: 1) Activities and practices in allogeneic transplantation, 2) legal frameworks and organizational structures in existence, 3) threats and responses identified through vigilance and surveillance programs, and 4) issues in xenotransplantation. Success of the GKT depends upon collaborations with many of the cellular therapy organizations and networks described in this chapter.

**Table 65-3.** Transplant Organizations and Their Associated Web Sites

| Organization                                                  | Web Site URL                                                                                                                                                                                        |
|---------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| National Marrow Donor Program                                 | <a href="http://www.marrow.org">http://www.marrow.org</a><br><a href="http://bioinformatics.nmdp.org">http://bioinformatics.nmdp.org</a><br><a href="http://www.agnis.net">http://www.agnis.net</a> |
| Caitlin Raymond International Registry                        | <a href="http://www.crir.org">http://www.crir.org</a>                                                                                                                                               |
| Gift of Life                                                  | <a href="http://www.giftoflife.org">http://www.giftoflife.org</a>                                                                                                                                   |
| Anthony Nolan Bone Marrow Trust                               | <a href="http://www.anthonynolan.org.uk">http://www.anthonynolan.org.uk</a>                                                                                                                         |
| ZKRD                                                          | <a href="http://www.zkrd.de">http://www.zkrd.de</a>                                                                                                                                                 |
| NetCord                                                       | <a href="http://www.netcord.org">http://www.netcord.org</a>                                                                                                                                         |
| World Marrow Donor Association                                | <a href="http://www.worldmarrow.org">http://www.worldmarrow.org</a>                                                                                                                                 |
| Center for International Blood and Marrow Transplant Research | <a href="http://www.cibmtr.org">http://www.cibmtr.org</a>                                                                                                                                           |
| American Society for Blood and Marrow Transplantation         | <a href="http://www.asbmt.org">http://www.asbmt.org</a>                                                                                                                                             |
| AABB                                                          | <a href="http://www.aabb.org">http://www.aabb.org</a>                                                                                                                                               |
| European Group for Blood and Marrow Transplantation           | <a href="http://www.ebmt.org">http://www.ebmt.org</a>                                                                                                                                               |
| Eurocord                                                      | <a href="http://www.eurocord.org">http://www.eurocord.org</a>                                                                                                                                       |
| Asia-Pacific Blood and Marrow Transplant Group                | <a href="http://www.apbmt.org">http://www.apbmt.org</a>                                                                                                                                             |
| International Society for Cellular Therapy                    | <a href="http://www.celltherapy.org">http://www.celltherapy.org</a>                                                                                                                                 |
| Worldwide Group for Blood and Marrow Transplantation          | <a href="http://www.wbmt.org">http://www.wbmt.org</a>                                                                                                                                               |
| Foundation for the Accreditation of Cellular Therapy          | <a href="http://www.factwebsite.org">http://www.factwebsite.org</a>                                                                                                                                 |
| Joint Accreditation Committee—ISCT and EBMT                   | <a href="http://www.jacie.org">http://www.jacie.org</a>                                                                                                                                             |
| Alliance for Harmonisation of Cellular Therapy Accreditation  | <a href="http://www.ahcta.org">http://www.ahcta.org</a>                                                                                                                                             |
| Health Resources and Services Administration                  | <a href="http://bloodcell.transplant.hrsa.gov">http://bloodcell.transplant.hrsa.gov</a>                                                                                                             |
| World Health Organization                                     | <a href="http://www.who.int/transplantation/en/">http://www.who.int/transplantation/en/</a>                                                                                                         |

## Summary

Many organizations are engaged in managing cellular therapies (Table 65-3). Most of them are currently supporting HSCT therapies, which use products from adult donors and umbilical cord blood donors. Examination of these organizations and their diverse and interactive roles gives a clear picture of the impressive level of activities needed to support a relatively mature but expanding field. As new cellular therapies such as the burgeoning field of regenerative medicine emerge, the complex functions needed to support them will be met in some cases by expanding the reach of the existing organizations and in others through the establishment of new ones.

## Disclaimer

The author has disclosed no conflicts of interest.

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# Hospital Transfusion Committee and Quality Assurance

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*Blood bank*, coined by Fantus,<sup>1</sup> was the term originally applied to that area of a hospital in which blood was stored in containers for transfusion to patients. The dynamic roles of the medical director and staff of this area in contemporary clinical practice make the term *transfusion service* more appropriate. This term better captures the responsibilities of ensuring safe transfusion practice and appropriate blood use. The transfusion service, in many instances, functions more as a trust fund than a bank. The US Food and Drug Administration (FDA) and the AABB have their own nomenclature for the terms *blood bank* and *transfusion service* as described later. The generic term *transfusion service* is used throughout this chapter to describe all hospital facilities that engage in storing blood components and providing them for transfusion.

The role of the transfusion service laboratory in performing pretransfusion compatibility testing has evolved dramatically from a matrix of elaborate protocols, including major and minor crossmatches and antibody detection tests performed at both room temperature and 37°C, to the present practice in many laboratories of performing a computer crossmatch when certain conditions are met for patients in whom no red cell alloantibody has been detected in current or previous testing.<sup>2,3</sup> At the same time, development of preoperative crossmatch guidelines and the introduction of testing only for ABO group, Rh type, and unexpected antibodies for patients unlikely to need transfusion have been implemented to conserve transfusion service inventories.<sup>4-7</sup>

The Clinical Laboratory Improvement Act and Amendments (CLIA) stipulate requirements for the qualifications of staff who perform or supervise the highly complex testing conducted within a transfusion service. AABB standards<sup>8(p3)</sup> and FDA current good manufacturing practice (cGMP) regulations also require that the transfusion service have a process for personnel training and competency evaluation.

Written standard operating procedures are required by the FDA in the *Code of Federal Regulations* (Title 21 CFR 606.160). A system must be in place to ensure process control for validation of processes and procedures, introduction and change of processes and procedures, proficiency testing, quality control, and the use of materials and other aspects of performance of procedures. This is also required by AABB standards and FDA cGMP regulations. A defined system of documentation and record retention is also required by both (21 CFR 606.100, 606.140, and 606.160). Further aspects of FDA regulations and accreditation requirements of the AABB, The Joint Commission, College of American Pathologists (CAP), and other accrediting organizations are as described later.

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## Role of the Medical Director

The role of the transfusion medicine specialist is to maintain a “central position in the flow of blood components and products” within the institution.<sup>9</sup> Consequently, the medical director of a transfusion service has three essential roles: administrative, clinical, and educational.

### Administrative

Of the three roles mentioned above, the administrative role is the most problematic, because most statutory and regulatory bodies do not mandate that the medical director be the administrative head of the blood bank and transfusion service. The FDA no longer requires that a “blood establishment” be under the direction of a designated, qualified person in all matters relating to compliance. Similarly, the AABB *Standards for Blood Banks and Transfusion Services*<sup>8</sup> requires that the transfusion service have a medical director with responsibility and authority for all medical and technical policies, processes, and procedures (including those pertaining to personnel and test performance) as well as for consultative services related to the care and safety of transfusion recipients. However, it does not require that overall executive management be under the control of the medical

director.<sup>8(p1)</sup> The Joint Commission also does not require that the overall direction of the laboratory be performed by a physician but does state that “a pathologist or physician qualified in immunohematology, hemotherapy, and blood banking directs blood transfusion services” (HR 1.15).<sup>10</sup>

Consequently, different organizational structures are possible, and the medical director, while retaining authority for all medical and consultative aspects of the transfusion service, may or may not be its overall administrative head. Depending upon the management structure of the laboratory, administrative authority that may lie outside the medical director's control includes responsibility for the operations, quality system, compliance with standards and regulations, and review of the quality system. Many transfusion services have a chief supervisor, who reports to a laboratory manager, who in turn reports to the executive management of the hospital. In this arrangement, the medical director is not in a direct reporting relationship to those responsible for laboratory administration. This often excludes the medical director from authority in personnel (other than policies and procedures), purchasing, budgeting, and other administrative matters. However, CLIA (1988) mandates that the director of a laboratory have responsibility for its functions. The regulation states that the laboratory director must be a doctor of medicine or doctor of osteopathy (42 CFR 493.1443).<sup>11</sup> This creates a situation in which the medical director has statutory responsibility without administrative authority. This dilemma must be solved with pragmatic cooperation between the administrators and the medical director.

## Clinical

The medical director of the transfusion service should work with the clinical staff and hospital administration regarding all aspects of blood transfusion. The director should be recognized as the specialist in transfusion medicine within the institution and serve as the primary resource in related matters. There are many ways to establish this role, but they typically require that the director develop and implement initiatives in a variety of areas. Within the blood bank, the director can bring his or her clinical knowledge to bear on decisions regarding the extent of pretransfusion compatibility testing, the investigation of alloantibodies, the length of time specimens are held for subsequent testing, and the selection of components for inventory (eg, type of fibrin sealant, timing of leukocyte reduction, type of platelet component, inventory of pathogen-inactivated components). Even if the medical director is not responsible for the overall quality assurance program, he or she must participate actively in any quality assurance program related to blood transfusion. As described later, the director represents the transfusion service on the hospital transfusion committee in the institution.

Outside of the laboratory, the medical director should provide expertise and develop policies and procedures that address all aspects of blood supply, blood administration, and adverse reactions. The director should participate in the formulation of institutional policies to ensure an adequate supply of blood to

meet patient needs, review of procedures for blood administration, development and implementation of procedures for perioperative blood conservation and reinfusion, and consideration of alternatives to blood component transfusion (eg, hematopoietic growth factors, blood substitutes when licensed). This provides leadership in shaping and standardizing transfusion practice, and may be done in conjunction with the hospital transfusion committee.

The director must ensure that appropriate processes for blood administration minimize the possibility of human error contributing to an adverse outcome. The AABB standards include accountability of the director for the care and safety of transfusion recipients.<sup>8(p1)</sup> A system for correct identification of patients is critical. Because most transfusions are performed by nurses, the transfusion service should work with the nursing services to ensure that their procedures are appropriate and clear and that appropriate education of the nursing staff occurs. Most hospitals require that two persons verify all of the appropriate information at the bedside before transfusion. The transfusion service director should plan an active role in reviewing and ensuring appropriate use of devices associated with transfusion, including filters and blood warmers. Policies regarding the addition of other fluids to blood components also should be reviewed by the director. In addition, CAP requires that the medical director be involved in resolving a system failure in blood administration (TRM.41770).<sup>12</sup>

The director is also responsible for evaluating all reported adverse events resulting from blood transfusions and making recommendations regarding future hemotherapy. The institution must have a procedure for ensuring that adverse events are reported promptly to a physician caring for the patient and to the transfusion service. The FDA, AABB, and CAP all have requirements regarding the evaluation and reporting of adverse effects of blood transfusion. The FDA requires that records be maintained of any reports of adverse reactions to blood transfusion and that a thorough investigation of each reported reaction be conducted. All transfusion services must report deaths confirmed as being caused by a transfusion to the Office of Compliance, Center for Biologics Evaluation and Research, by telephone (301-827-6220); fax (301-827-6748) or email (fatalities2@fda.hhs.gov) as soon as possible and by writing within 7 days, in accord with 21 CFR 606.170.<sup>13</sup> The AABB requires that a transfusion service have a process for the detection, reporting, and evaluation of suspected complications of transfusion and that all suspected transfusion complications are evaluated and reviewed by the director.<sup>8(p94)</sup> The CAP requires that all transfusion reactions or incidents be reported immediately to the laboratory (TRM.41750) and that documented procedures exist for actions to be taken in the event of a transfusion reaction (TRM.41700).<sup>12</sup> An interpretation by the director of all hemolytic transfusion reaction investigations must be recorded in the patient's chart (TRM.42050).<sup>12</sup>

The director should also create a variety of opportunities for interaction with the clinical staff and should market his or her

availability for consultation. Requested consultations typically concern massive transfusion, therapeutic apheresis, refractoriness to platelet transfusion, unexpected perioperative bleeding, and invasive procedures possibly necessitating preprocedure platelet or plasma transfusion. More informal consultation opportunities arise in response to transfusion orders that appear excessive, inappropriate, or unusual. Here, interaction with ordering physicians serves the dual purpose of shaping improved patient care while informing them of the expertise available from the transfusion service. Each facility should develop its own list of situations in which contact is initiated according to the scope of services of the facility. These can include requests for more than one platelet transfusion, more than 6 units of Fresh Frozen Plasma (FFP) per day for the same patient, a granulocyte concentrate, fresh blood, or a customized blood component (eg, a cytomegalovirus-reduced-risk or irradiated product). If inventory issues dictate exceptions to practices (eg, an Rh-negative patient will receive Rh-positive red cells), the medical director should call the patient's physician to explain the situation and suggest appropriate patient follow-up care. Directors of transfusion services also should speak to a patient's physician when transfusion will be delayed because of the presence of red cell alloantibodies or for any other reason.

The director should review the laboratory and clinical data to assist in ordering of diagnostic tests and in blood transfusion management, particularly for patients who have received a large number of Red Blood Cell (RBC) units without other components. Such review of laboratory information for patients receiving blood components also provides the director with other opportunities for informal consultation. For example, the director may note refractoriness to platelet transfusions and offer prompt advice for diagnosis and management. Drug-induced platelet antibodies are not always detected by clinicians. A review by the transfusion service director of patients who do not respond to platelet transfusions is likely to uncover such occurrences.

Advice also can be provided as the result of established testing algorithms within an institution. Approved by the medical staff, such reflexive systems can meet compliance requirements and lead to interpretive, billable reports that provide for efficient clinical care. Bleeding and thrombotic disorders are complex clinical situations that can be clarified more efficiently through directed laboratory testing algorithms. Red cell alloantibody identifications also lend themselves to interpretations by the transfusion service director regarding the likelihood of delays in blood availability and the potential clinical significance.

Each engagement between a transfusion service director and a clinician provides opportunities not only for improving an individual patient's care but also for forging relationships that will lead to future consultation. Cooperative efforts among physicians from different disciplines are important to improve the use and ongoing evaluation of blood components.<sup>14,15</sup> For example, to ensure the appropriate use of FFP, the cooperative effort of hematologists knowledgeable in coagulation, treating physicians,

and the transfusion service director often is useful. Similarly, the director may consult with hematologists to establish a threshold for prophylactic platelet transfusions that may be lower than current practice. Patients would be exposed to fewer blood components, resulting in improved patient care, institutional cost savings, and conservation of the blood supply.

## Education

The heightened awareness of patients and physicians regarding the risks of blood transfusion therapy has made the role of the transfusion service director a more public one. This prominence has influenced attending physicians to be receptive to advice and education from the director.

The transfusion service director serves as the principal educator of clinicians and house staff in blood transfusion practice. The director must be aware of the contemporary literature on the use of blood components, including consensus conference statements, practice guidelines, and emerging standards of practice. The director can educate the clinical staff on the availability of newly introduced blood components and pharmaceuticals (eg, pathogen-inactivated blood components, fibrin sealant), and serve as an aggressive patient advocate for the introduction of these products for improved patient care.<sup>16</sup> Interactions between the transfusion service director and the attending physician should be designed as educational and nonconfrontational and should be conducted in the spirit of mutual patient advocacy. The available forums for such educational opportunities are numerous and include informal consultations as noted above as well as more formal teaching opportunities such as grand rounds or continuing education lectures given to hospital staff actively involved in blood transfusion practices.

The foregoing activities provide for improved care, reduced costs, and shorter hospital stays. The quiet accomplishment of a laboratory director in obtaining an adequate blood supply and making this available for transfusion may not always be recognized as evidence of the director's contribution to patient care. Although the director may be held responsible for the failure to achieve these objectives, there may be a presumption that little effort or training was required to accomplish them. The transfusion service director, therefore, must strive not only to add value to medical care but also to educate others about this important effort.<sup>14</sup>

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## Quality Assurance

### Historical Overview

As long ago as 1667, Denis' historic transfusion was subjected to extensive review.<sup>17</sup> Concern about abuse of blood was published in 1936.<sup>18</sup> In 1951, the Ministry of Health in England called for the review of blood use at medical staff meetings. In the same year, an editorial in the *New England Journal of Medicine* noted the inappropriateness of some transfusions.<sup>19</sup> A 1953 audit study concluded that only 241 of 290 transfusions were indicated.<sup>20</sup>

Additional publications between 1956 and 1960<sup>21-26</sup> echoed this message, emphasized the physician's potential liability if an unnecessary transfusion were administered, and suggested the usefulness of audits. In 1961, The Joint Commission on the Accreditation of Hospitals and other experts emphasized the need to review transfusion practices.<sup>27,28</sup>

By 1962, indications for blood transfusion were being included in the bylaws of hospitals.<sup>29</sup> In the same year, the Joint Blood Council called for a review of transfusion practices by a medical staff committee.<sup>30</sup> Several authorities recommended the establishment of a hospital transfusion board to review transfusion practices and called attention to the educational function such a committee would serve.<sup>31-34</sup> A 1965 study showed that a large number of transfusions were inappropriate and could not be justified by the ordering physicians.<sup>35</sup> In 1974, 14 of 46 Minnesota hospitals responding to a survey had procedures for reviewing indications for blood transfusion; among them, 25 had some form of transfusion committee.<sup>36</sup>

With the advent of the AIDS epidemic in the 1980s these existing institutions for review of blood utilization acquired new importance as the infectious hazards of blood transfusion became increasingly evident. New transfusion guidelines provided more stringent criteria for transfusion based on available evidence of transfusion efficacy.<sup>37-39</sup> Over the next several years, the entire blood community focused on reducing the risks of viral transmission, not only for human immunodeficiency virus (HIV), but for several other important pathogens with remarkable results.<sup>40</sup>

## Process Control

The disappearance of viruses as a major transfusion hazard did not end the increased emphasis on improving safety. The concept of a "zero-risk" blood supply and the application of the precautionary principle to transfusion medicine as a result of the AIDS epidemic encouraged the extension of industrial management theory into clinical laboratories; and quality assurance programs derived from industrial practices have been increasingly applied to clinical medicine.<sup>41,42</sup> This is not to say that numerous safeguards were not already in place. AABB standards were first enacted in 1958 to prevent untoward events associated with blood transfusions, long before the application of industrial quality systems to blood banking. However, industrial methods of continuous quality improvement are an important addition to blood safety because fatal errors can occur at any point in the blood supply chain from donor to recipient. At the blood collecting agency, errors in collection, testing, component processing, labeling, storage, and transport all can result in a misidentified or substandard product. The transfusion service must have policies and procedures in place for proper patient identification, sample testing, blood administration, and overall transfusion practice. In order to have a total quality system, institutions must have systems that, on the front end, reduce opportunities for error; and on the back end, appropriately evaluate and manage those errors that inevitably occur.

On the front end, the quality system<sup>43,44</sup> is a program implemented to ensure that all parts of the processes of an enterprise work together to produce the expected output. Quality management is the totality of activities planned and performed to provide confidence that all procedures are functioning as expected. A quality management program must be designed and implemented to ensure that procedures are consistently performed. Elements of a quality program include quality control procedures and standards for personnel, facilities, procedures, equipment, and record-keeping. Audits are an integral component of a quality program. As a matter of good practice, those responsible for quality assurance should report to senior management and not to operations personnel.

Total quality systems have been required for blood banks and transfusion services since 1975 when the FDA incorporated cGMP into the *Code of Federal Regulations*. Although the regulations were aimed primarily at blood centers, many of the provisions apply to transfusion services as well. AABB *Standards for Blood Banks and Transfusion Services* apply equally to member blood centers and transfusion services. These standards provide a blueprint for a total quality system, breaking operations down into 10 Quality System Essentials (QSEs) listed in Table 66-1. These 10 QSEs require that there be policies, processes, and procedures in place governing each of the 10 areas outlined in the essentials; exactly how the AABB standards are implemented is determined by the individual institution. The AABB quality system is discussed in detail below.

The development of a quality system in transfusion services allows certification by the International Organization for Standardization (ISO) quality management system that has been used by many businesses since 1986.<sup>45</sup> A few blood and tissue centers have received ISO 9002 accreditation. The ISO 9002 standards provide guidance in the development and implementation of an effective quality management system. It is not specific to any particular product or service but can be used generically by all enterprises. Complying with ISO 9002 standards does not mean that every product or service meets specific requirements. It means only that the quality system in use is capable of meeting them. The ISO standards basically require that an enterprise document its processes and then follow them as stated.

In general, all quality systems strive to identify process errors and defects and then eliminate them with sustainable changes through redesign of processes. In these programs, the staff of an institution strives to create cycles of continuous improvement in the course of designing, measuring, assessing, and refining work processes. Existing processes are carefully described and the outcomes are measured. The measurable outcome of the process monitored must be defined at the outset. Data are then analyzed and opportunities for improvement are identified. Changes that should improve the process and eliminate outcome variability are implemented and the outcome is again measured. The goal is process control: the collective set of actions taken to minimize variability in a process, thereby minimizing

**Table 66-1.** AABB Quality System Essentials

|                                                                     |                                                                                                                                                                                                                                                                                                                                        |
|---------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Organization</b>                                                 | A structure that clearly defines and documents those responsible for the provision of blood components and services and the relationship of individuals responsible for key quality functions.                                                                                                                                         |
| <b>Resources</b>                                                    | Policies, processes, and procedures to ensure the provision of adequate resources to perform, verify, and manage each of the activities of the transfusion service.                                                                                                                                                                    |
| <b>Equipment</b>                                                    | Policies, processes, and procedures to ensure that calibration, maintenance, and monitoring of equipment conform to standards and applicable requirements.                                                                                                                                                                             |
| <b>Supplier and customer issues</b>                                 | Policies, processes, and procedures to evaluate the ability of providers to meet requirements consistently.                                                                                                                                                                                                                            |
| <b>Process control</b>                                              | Policies and validated processes and procedures to ensure the quality of the blood components, tissue, and services provided and that procedures are conducted under controlled conditions.                                                                                                                                            |
| <b>Documents and records</b>                                        | Policies, processes, and procedures to ensure that documents are identified, reviewed, approved, and retained and that records are created, stored, and archived in accord with retention policies.                                                                                                                                    |
| <b>Deviations, nonconformances, and adverse events</b>              | Policies, processes, and procedures to ensure the capture, assessment, investigation, and monitoring of deviations from, or of failures to meet, specified requirements. Definition of responsibility for review and authority for the disposition of nonconforming products and for appropriate reporting of deviations and failures. |
| <b>Assessments: internal and external</b>                           | Policies, processes, and procedures to ensure that external assessments are obtained at defined intervals and that internal assessments are conducted.                                                                                                                                                                                 |
| <b>Process improvement through corrective and preventive action</b> | Policies, processes, and procedures for data collection, analysis, and follow-up of issues that require corrective or preventive action.                                                                                                                                                                                               |
| <b>Facilities and safety</b>                                        | Policies, processes, and procedures for the provision of a safe and adequate workplace.                                                                                                                                                                                                                                                |

variability of outcome. Such a data-driven approach, in which multiple cycles of outcome assessment are measured, results in overall improvement of the process being measured and uniformity of outcome.<sup>46</sup>

As an example of improved process control, the instances of unacceptable turnaround time for release of a blood product can be reduced simply by reducing the variability in the time required to process a request for blood, even if the mean time is not changed. When variance is reduced and consistency improved, control of a process is demonstrated, and benefits for interrelated systems are generated. For example, if there is minimal variability in turnaround times for release of blood products, transportation services and clinical services can coordinate their activities with this expectation.

However, a reasonable approach to documentation should be maintained. No purpose is served by collection of data without analysis and interpretation and without using the interpretation to improve a process. Once a process has been shown to result in minimal variation in outcome and to meet expectations, it is reasonable to consider whether requirements can then be raised. In this way, an effective quality assurance program can directly lead to increased operational efficiency. Again using the example of unacceptable turnaround times, once process control improves variability in turnaround time, it is reasonable to reexamine the process to see whether an absolute reduction in turnaround time could now be attained.

Any quality program must include a commitment from management to build quality into the system rather than just rely on inspection of a final product or service. There should be a spirit of cooperation between quality assurance and operations

personnel. The goal is continuing improvement that will translate into improved patient care, regulatory and accreditation compliance, and good business practices. Quality assurance plans must be manageable and clearly understood by the all hospital staff as well as by individual workers charged with compliance. Finally, for any hospital quality assessment program to work successfully, physicians must give priority to the process, and hospital administration must make the necessary resources available to ensure its development, implementation, refinement, and continuation.

### Error Management

Although the safety of the blood supply has increased dramatically, the overall safety of blood administration has not. Transfusion error continues to be a great risk, and most errors result from preventable human mistakes. Fully 70% of these untoward events occur outside of the blood bank laboratory, and the most common error is failure to properly identify a patient during the transfusion process—either at the time of sample collection or at the time of transfusion.<sup>47,48</sup> Several studies have documented miscollection of samples, the so-called “wrong blood in tube” error, at a rate of approximately 1 in 2000.<sup>49–51</sup> In one study, samples with detectable labeling errors were 40 times more likely to have been miscollected than samples that were properly labeled.<sup>51</sup> Comparison with prior records prevents many ABO-incompatible transfusions; however, safety checks in the blood bank cannot detect miscollected samples where there is either no prior blood type on record or where the blood in the tube matches the recipient blood type by chance. (For this reason, some hospitals require that two samples be drawn at different times to confirm blood type on patients

without historical records.) One study noted that approximately 1 in 3400 samples could be identified as miscollected on the basis of ABO discrepancy, suggesting that the rate of miscollection is much higher.<sup>52</sup>

The most common error in the transfusion chain, however, is failure to properly identify a patient at the time of blood transfusion. A recent review from the United Kingdom hemovigilance program (the Serious Hazards of Transfusion, or SHOT, program), cited failure to perform a proper bedside check as the proximate cause for 27% of reported mistransfusions.<sup>48</sup> Another study found that in 25% of audited transfusions, there was no comparison of the patient's wristband with the blood tag, an omission of a vital safety step.<sup>53</sup> Further, errors in identification may actually be increasing. Reports document a significant increase in problems with identification of patients, samples, and units.<sup>53,54</sup> Patient identification has been at the heart of errors in other areas of hospital practice, most notably delivery of medications, and because of these problems, The Joint Commission has made proper patient identification its primary safety goal.

At present, the risk that a patient will receive the wrong unit of blood in the United States is in the order of 1 in 12,000 to 1 in 19,000, approximately 100 times the risk of acquiring hepatitis C virus or HIV by means of blood transfusion.<sup>55,56</sup> Reports on mistransfusion from other countries have quoted remarkably similar rates.<sup>57–59</sup> The actual transfusion-related death rate due to ABO hemolytic transfusion reactions lies somewhere between 1 in 1,000,000 and 1 in 2,000,000.<sup>55,56,60</sup> Similarly, the SHOT program reported that the likelihood of receiving an ABO-incompatible unit at 1 in 100,000.<sup>47</sup> Again, assuming a fatality rate of between 5% and 10% for an ABO-incompatible transfusion, this translates to a death rate of between 1 in 500,000 and 1 in 1,000,000.

Although 70% of transfusion errors occur in clinical areas of the hospital, the remaining 30% of mistransfusions result from laboratory error.<sup>47,55</sup> Not surprisingly, as with transfusion errors elsewhere, clerical mistakes such as selection of the wrong sample for testing explain most of these errors. A disproportionate percentage of laboratory errors occur outside of peak hours of operation. The SHOT program reported that 40% of laboratory errors occurred during periods where only 20% of the workload was processed. Commentators have suggested that this discrepancy between workload and error rate can be accounted for by several factors, including decreased staffing during off-hours, relatively inexperienced staff, and the pressure of medical emergencies occurring at times when staffing is less than optimal.<sup>47</sup>

Several countries have instituted measures to address these noninfectious serious hazards of transfusion. Hemovigilance refers to comprehensive surveillance systems that monitor the entire transfusion process from beginning to end by collecting data on adverse events associated with transfusion. The aim is to collect and assess information obtained from adverse events so that recurrence can be prevented. In effect, hemovigilance is the introduction of process control measures to the entire transfusion process. All member states of the European Union are required to have hemovigilance systems (EU directive 2002/98/EC),

although the form of these systems can differ. Some countries (for example, France) have mandatory programs where all serious adverse hazards must be reported.<sup>61</sup> Others, such as England, have centralized, nationalized programs where reporting is voluntary.<sup>47,48</sup> Clearly, the introduction of hemovigilance systems results in increased reporting of noninfectious transfusion complications. For example, introduction of a notification system in Quebec resulted in a dramatic increase in the reporting of such incidents.<sup>62</sup> Similarly, the reports of incorrect transfusion of blood components increased yearly with the implementation of the SHOT program in England, but were coupled with a noticeable downward trend in the number of ABO-incompatible transfusions several years after the introduction of the hemovigilance system.<sup>47</sup>

The United States began implementing a hemovigilance program in 2008. The voluntary, nonpunitive, Internet-based system was created through a public-private partnership involving the Centers for Disease Control and Prevention, the AABB, and other stakeholders. The FDA requires that errors or accidents affecting the safety, quality, integrity, purity, or potency of a blood product be reported, and this requirement extends to both licensed blood establishments and transfusion services (CFR 606.171).<sup>13</sup> However, this statutory authority extends only to the blood product itself, and does not permit FDA oversight of the actual transfusion episode. Although the FDA Medwatch program provides a voluntary venue for reporting adverse events, most hospitals do not voluntarily report errors to this particular program. Hence, the FDA's purview extends only as far as the laboratory door, and there is no requirement to report a patient misidentification error unless it results in a death. (A transfusion-related death, however, must be reported to the CBER division of FDA within 24 hours.) To give an example of this regulatory lacuna, the following example suffices: failure to irradiate a unit of RBCs that was so ordered results in an FDA reportable error. Transfusion of the same unit to the wrong patient does not result in a reportable error unless the patient dies.

Clearly, the continuing noninfectious risks of blood transfusion coupled with the lack of a national hemovigilance program constitutes a strong argument for hospital-wide efforts at improving the process of transfusion safety. There has been inadequate commitment of financial resources directed toward improving safety in this area, although The Joint Commission's safety goal of proper patient identification may change this. The introduction of process control to the transfusion service itself has not eliminated the major risks of a blood transfusion because most of the steps involved in the transfusion of a unit of blood lie outside the direct control of the transfusion service. Given the number of steps required to correctly identify a patient at the time of phlebotomy, properly label a blood sample, and properly coordinate identification of a specific patient with a specific unit of blood at the time of transfusion, it is not surprising that errors occur. Staff may not understand the importance of proper patient identification in the transfusion setting, because similar identification is not required for other equally common hospital

procedures. Consequently, there is a strong inclination to either ignore the identification procedures or to circumvent them, because they take time and are difficult to perform correctly. Further, workload on busy hospital services is more difficult to manage than laboratory workload, and therefore is less amenable to process control. A patient's condition can change dramatically in a matter of minutes. Hospital staff must respond to medical emergencies and unanticipated events. Therefore, in an emergency, there is a tendency to truncate necessary safety procedures in order to provide rapid care, and as a result, errors occur. Studies show that fatal transfusion errors are most likely to occur in areas of the hospital where emergencies occur frequently such as the operating room and intensive care units.<sup>47,63</sup>

Error management must be a comprehensive system for identifying all errors, analyzing those errors, and determining the best system-wide approach to ensure that similar errors do not recur. An effective error management system should identify all "near-miss" errors as well as errors that cause actual harm to a patient. The concept of near miss is taken from industries where errors can cause catastrophic results. Near-miss errors are errors that do not cause actual harm, but could have had the circumstances been slightly different.<sup>64</sup> One study suggested that near miss errors occur three to 300 times more commonly than errors that cause harm.<sup>65</sup> As such, they provide a window into systems problems, and also provide the opportunity to analyze and restructure so as to prevent an actual medical catastrophe. An example of a near miss is as follows. Nursing personnel fail to properly identify a patient, resulting in mistransfusion, but the unit transfused is group O blood. The patient suffers no harm, but the error provides an opportunity to examine the overall transfusion process and make changes before a similar event results in a serious ABO-incompatible transfusion.

One other important concept fundamental to a well-functioning error management system is that human errors are inevitable, and most are unintentional. Therefore, error management should be conducted by means of detecting and solving system problems rather than placing blame and punishing individual workers for making a mistake. Such punitive management of error discourages reporting and decreases the potential for improvement. It is vital that staff be encouraged to report errors, because only with comprehensive detection of all errors can effective change be initiated. Once errors are detected, careful root cause analysis provides insight into system-wide dysfunctions and permits the development of system-wide changes.<sup>66</sup> One such system specific for blood transfusion is the Medical Event Reporting System developed under the auspices of the National Heart, Lung, and Blood Institute in 1995 and in use at several hospitals throughout the country. The system aims for exhaustive detection of errors including examination of quality assurance records, chart audits, and direct reports of errors by staff. Each event is assigned a numeric value based on the potential severity of harm and the likelihood of recurrence. Only those errors that reach the appropriate numeric threshold are subjected to a complete root cause analysis. Additionally, each error is

classified using a predetermined set of event codes and entered into a computer database so that patterns and trends can be identified. The results of the root cause analysis are then used to institute changes if necessary to prevent a recurrence.<sup>67</sup>

Technology can also be used to address transfusion error. As noted above, the majority of errors involve failure to follow proper patient identification procedures. Two of the weak points in the transfusion process—namely, patient sample collection and blood administration—can be subjected to technology that prevents identification errors. Both barcode and radio frequency identification (RFID) technologies can automatically read encoded information on the patient's wristband and on an accompanying blood bag tag. In the case of sample collection, a phlebotomist merely scans a patient's wristband, and produces a label at the bedside from a hand-held printer. As long as the tube is also labeled at the bedside, the problem of "wrong blood in tube" disappears. In the case of blood administration both the wristband and the bag tag are scanned, and again, a handheld device determines whether the two sets of information match. Although this technology is in its infancy, systems have been developed and are now being used in several hospitals around the world.<sup>68–70</sup>

### Improving Transfusion Practice

An important component of overall quality assurance in transfusion is ensuring that each transfusion that occurs is a necessary and appropriate medical therapy. Most clinicians have no formal training in transfusion medicine. Consequently, their practice is based on what they learned during training. Therefore, much of what they know is either outdated, or based on the transfusion "mythology" extant in their training hospital. One study documented that well over half of physicians given various clinical scenarios would have prescribed transfusion therapy that was inappropriate.<sup>71</sup> A number of approaches have been used successfully to improve clinical transfusion practices. Toy<sup>72</sup> has summarized five processes for which there is published evidence of success.

One approach is to give lectures, because written materials alone have been shown to be ineffective in changing medical practice.<sup>73</sup> Although many physicians may be reached simultaneously, it may be difficult to have a durable impact on transfusion practice. Publications on this approach have documented a decrease in inappropriate use of FFP from 53% to 22% through an educational conference program,<sup>74</sup> a 75% decrease in the total number of red cells transfused in an obstetrics and gynecology service,<sup>75</sup> and a 46% decrease in use of FFP over a 2-year period.<sup>76</sup> The proportion of patients donating autologous blood decreased from 53% to 26%, the overall transfusion rate decreased from 10% to 7%, and the allogeneic transfusion rate did not change in a program that decreased unnecessary autologous collections without increasing the number of allogeneic transfusions.<sup>77</sup>

A second approach is for a specialist in transfusion medicine to meet one-on-one with physicians. Although this is



time-consuming, it can be particularly effective. Brief face-to-face conferences with surgeons reduced the proportion of transfusions not in compliance with criteria from 40% to 24%. Surgeons who participated in the study decreased their transfusion trigger significantly whereas control physicians did not.<sup>78</sup> The use of autologous blood donation increased with visits to surgeons. No changes occurred in control hospitals where no visits were made. There was no increase in inappropriate use of autologous blood donations in study hospitals for procedures that required no preoperative blood order.<sup>79</sup> Review of transfusions 1 day after administration with discussions with the ordering physician led to a reduction from 3.2% to 0.5% in the number of transfusions that failed to meet auditing criteria.<sup>80</sup> In this study, the rate of unjustified transfusions decreased from 1.4% to 0% during a 1-year audit.

A third approach is to make patient rounds on a regular basis. This approach is time-consuming but directs physician attention to a particular patient who needs treatment and therefore may be particularly effective. A 77% reduction in the use of FFP over a 2-year period after implementation of a next-day review accompanied by an educational program has been demonstrated.<sup>81</sup> The number of units transfused to patients with a hematocrit greater than 30% decreased from 30% to 10% when daily rounds were made.<sup>82</sup>

A fourth approach is to review each transfusion order before the blood component is issued. This prospective review can be highly effective in improving transfusion practices. One mechanism for implementing this process is to have physicians select the indication for transfusion from a computerized order menu or by checking manually a transfusion order request form. Requests can also be reviewed by blood bank physicians and staff in conjunction with available laboratory data. Such a prospective review of blood utilization resulted in a significant reduction of allogeneic donor exposure in one study.<sup>83</sup> One paper<sup>84</sup> described how prospective audit of platelet transfusions, in which a substantial number of orders were initially denied or modified, resulted in significant improvement in platelet-ordering practices and a 56% reduction in platelet use over a 3-year period. An audit of platelet use followed by modified practice guidelines applied prospectively to requests for platelets led to a 14% decrease in the number of platelet units transfused during the following year.<sup>85</sup> A mandatory pretransfusion approval program resulted in a 33% decrease in FFP transfusions.<sup>86</sup> A decrease from 28.6% to 27.7% in the hematocrits of persons receiving transfused occurred, as did a reduction in the number of transfusion orders requiring review, after computerized screening of blood transfusion requests was implemented.<sup>87</sup> A significant reduction in inappropriate platelet and FFP transfusions occurred after introduction of a new component request form that included transfusion guidelines.<sup>88</sup> A significant decrease in inappropriate red blood cell, platelet, and FFP transfusions after the request form was modified to include indications for transfusion and relevant clinical and laboratory data was shown in another study.<sup>89</sup>

A fifth approach to improving transfusion practices is to develop and implement algorithms. Success of an intraoperative algorithm for transfusion in cardiac surgery has been reported.<sup>90</sup>

Retrospective review of charts, while satisfying some accreditation requirements, has not been shown conclusively to influence transfusion practices when used as a single tool. Retrospective analyses can, however, augment utilization review when combined with other approaches. This activity, along with other approaches to reviewing the process of transfusion, and the development of review criteria are addressed later, in the section on the transfusion committee.

The combination of approaches has been successful in improvement of transfusion practice. Reviewing clinical and laboratory data for all transfusions with follow-up consultations substantially reduced the number of transfusions of red cells, platelets, FFP, and cryoprecipitate over a 4-year period.<sup>91</sup> A 52% decrease in use of FFP occurred after implementation of a retrospective utilization audit, an educational program, a new FFP request form that required the ordering physician to indicate the reason for the request, and pathologist approval in cases lacking documentation of abnormal results of coagulation studies.<sup>92</sup> The establishment of transfusion guidelines based on national standards accompanied by the implementation of a monitoring system that required physicians to record medical indications for all nonemergency transfusions and followed by selective retrospective auditing resulted in a 35.9% decrease in allogeneic donor exposures over a 3-year period.<sup>93</sup> In a novel approach, a quality assessment program that permitted oversight of transfusionists' practices resulted in a decline from 50% to nearly zero in the percentage of transfusions at variance from institutional blood administration policy.<sup>71</sup>

Educational strategies should be aimed at the attending physician who has the final responsibility for transfusion orders.<sup>94</sup> One study showed that attending physicians had lower knowledge scores than did residents but exhibited more confidence in what they knew. Resident ordering practices were heavily influenced by the wishes of attending physicians. Most residents who answered the survey stated they had ordered transfusions they had judged unnecessary because a more senior physician had suggested that they do so. Multi-institutional audits also serve as useful benchmarking tools.<sup>95</sup>

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## Regulatory and Accreditation Requirements

A number of regulatory agencies and accrediting organizations in the United States have developed standards regarding the quality assessment and improvement of blood transfusion practices.

### Food and Drug Administration

#### FDA and Transfusion Services

The FDA considers establishments that perform certain activities that it defines as manufacturing steps to be hospital blood banks, which are required to register annually using form FDA2830.

A hospital blood bank is an entity that routinely collects or processes whole blood or blood components. These components may be collected by means of apheresis or prepared from whole blood. Processing includes freezing, deglycerolizing, washing, irradiating, rejuvenating, or removing leukocytes from components. Hospital laboratories that solely prepare RBCs or Recovered Plasma, pool Platelets or Cryoprecipitated AHE, or issue bedside leukocyte reduction filters are considered hospital transfusion services and are not required to register [21 CFR 607.65(f)].<sup>13</sup>

Because blood and blood components are drugs under the Federal Food, Drug, and Cosmetic Act, the FDA cGMP regulations (21 CFR 210 and 211) apply to the manufacture of these products.<sup>96</sup> In addition, cGMP regulations for blood and blood components exist in 21 CFR 606.<sup>13</sup> All of these regulations apply to FDA-defined hospital blood banks. FDA registration allows the agency to plan and perform routine cGMP inspections.

The *Code of Federal Regulations* requires a quality program. In 42 CFR 493.1701, it is mandated that “each laboratory establish and follow written policies and procedures for a comprehensive quality assurance program . . . The laboratory’s quality assurance program must evaluate the effectiveness of its policies and procedures; identify and correct problems . . . All quality assurance activities must be documented.”<sup>11</sup> In 1995, the Center for Biologics Evaluation and Research produced a guideline to assist establishments in developing quality programs in accord with applicable regulations.<sup>97</sup>

Although the FDA does not routinely inspect hospital transfusion services, these services also engage in manufacturing in the view of the FDA, because compatibility testing, blood storage, labeling, and record-keeping are considered steps in the manufacturing process. Thus, transfusion services are also subject to cGMP regulations. Inspection of hospital transfusion services is overseen by the Centers for Medicare and Medicaid Services (CMS) through a 1980 memorandum of understanding with the FDA that addresses inspection of these establishments. In an effort to reduce duplication of inspections, it was agreed that inspection of hospital transfusion services that are approved for Medicare reimbursement and that engage in compatibility testing but that neither routinely collect nor process blood components would be subject to inspection by the CMS. This agreement pertains to responsibility for inspection only. No statutory authority transferred between the agencies. As part of the agreement, the CMS adopted FDA regulations in 21 CFR 606 titled “Current Good Manufacturing Practice for Blood and Blood Components” and 21 CFR 640 titled “Additional Standards for Human Blood and Blood Components.”<sup>13</sup> These are the FDA requirements that have been incorporated into the CLIA regulations. Observations made by the CMS may be communicated to the FDA, which can then directly inspect a hospital transfusion service.

All transfusion services, registered or unregistered and regardless of FDA nomenclature, must also comply with the regulations in 42 CFR 493 in accord with CLIA 1988.<sup>11</sup> For the purposes of CLIA certification, CMS retains responsibility for inspection of all transfusion services.

Many transfusion services may not be surveyed directly by the CMS. Some are in an exempt state or have been accredited by an organization that has been granted deemed status.

### FDA and Transfusion Service Error Reporting

Both registered and unregistered blood establishments, including transfusion services, must report errors and accidents in manufacturing to the FDA. Manufacturing is defined as including “testing, processing, packing, labeling, storage, holding, or distribution” of both licensed and unlicensed blood components. Errors and accidents (termed *biologic product deviations*) and unexpected events in manufacturing that can affect the safety, purity, and potency of a product are deemed reportable (21 CFR 606.171 and 21 CFR 600.14).<sup>13</sup> The FDA has devised a form, FDA3486, for reporting these deviations. The requirement to report applies only to manufacturing errors and not to transfusion errors occurring in clinical areas outside of the transfusion service.

Deviations must be reported only if the product was distributed. This is defined as having left the control of the establishment. If the product was not distributed, the incident still must be recorded in internal records [21 CFR 606.160(b)(7)(iii)].<sup>13</sup> If the product was distributed, a report must be submitted to the Center for Biologics Evaluation and Research within 45 calendar days from the date information is acquired that reasonably suggests a reportable event occurred. The incident must also be recorded [21 CFR 606.160(b)(7)(iii)] and investigated [21 CFR 606.100(c), 211.192 and 211.198].<sup>13,96</sup> The FDA has stated that the purpose of this reporting system is to provide early warning of faulty processes as an indicator for potentially immediate problems that may be related to recalls and as surveillance for improving training and establishing guidance.

The deviation or unexpected event must occur in the facility or another facility under contract with the controlling facility. If a facility under contract to the hospital blood bank or transfusion service is responsible for a deviation, the hospital blood bank or transfusion service is responsible for reporting the problem if the product is distributed. The contract facility must perform an investigation but is not required to report. For example, if a test laboratory under contract to a hospital blood bank fails to provide viral marker testing and the unit is subsequently distributed, the blood bank must report this. If a transfusion service discovers that a unit is mislabeled with an extended outdate, the transfusion service must notify the blood center responsible for reporting to the FDA. The transfusion service would report this incident only if it further distributed the unit without correcting the label.

Deviations and unexpected events occurring within the facility or a facility under contract must be reported if they may affect the safety, purity, or potency of both licensed and unlicensed products that have been distributed. However, as noted above, an error occurring after a product has left the facility need not be reported. Examples of events that would not require a report include a unit not being held at the appropriate temperature

before transfusion after release from the blood bank, transfusion of a unit to the wrong patient, or failure by hospital staff to use a filter issued by the transfusion service. Reportable, unexpected events may occur even if all established procedures are followed within the transfusion service itself. An example of this would be a patient sample used for compatibility testing that was collected from the wrong patient.

Not reporting an event to the FDA within 45 days in and of itself constitutes a nonreportable deviation. A record-keeping deviation, such as failure to include the signature of the person preparing the unit in component preparation, would not be reportable because it would not affect the safety, purity, or potency of the product. A unit labeled with a shortened expiration date would also not be reportable, nor would a unit drawn too soon after the last donation. It would not be a reportable event if an allogeneic unit were issued when autologous blood was available. However, a unit labeled with an extended expiration date would be a reportable deviation. In summary, a deviation or unexpected event is reportable if all of the following criteria are met:

- It was associated with manufacturing.
- It occurred in the facility or at a contract facility.
- It may have affected the safety, purity, or potency of the product.
- The facility had control over the product.
- The product was distributed.

### AABB

The AABB QSEs are consistent with FDA quality assurance guidelines and ISO 9002 standards. The intent of the QSEs is to provide assurance that quality principles are applied consistently throughout the entire enterprise, not just within specific operational areas. Use of the QSEs affords any transfusion service a guideline for organizing its activities and documentation. The use of audits for systems and processes provides an opportunity for considering each aspect of an activity and for influencing the quality of activities outside the immediate control of the transfusion service. Implementation of a quality management system provides for the routine capture of errors and accidents, some of which are required to be reported to the FDA. The activities of a quality system involve data collection and analysis to select processes for internal audits. These internal audits provide information necessary to make process improvements to prevent errors.

Although not required by the AABB or others, a quality manual provides a convenient means of ensuring that a transfusion service is meeting all the requirements of the QSEs. One such manual has been described and made available.<sup>98</sup> This manual includes a matrix that links AABB QSEs to source documents within the institution that demonstrate compliance. Existing procedure manuals can still be used.

The AABB assessment incorporates evaluation of the quality system at an institution and of each operational system. The quality system assessment is based on the same criteria for every facility. The operational systems, however, are identified by the

activities performed within an individual facility. The AABB standards use the quality management system as a framework.

The AABB accreditation program has deemed status under the regulations of CLIA to perform on-site assessments to meet CMS requirements. This status means that the CMS has determined that the AABB accreditation process provides assurance that facilities meet or exceed conditions required by federal law and regulations. A laboratory accredited by the AABB that designated AABB as its CLIA provider does not need to be inspected routinely by the CMS. However, these facilities are subject to validation surveys and surveys performed in response to complaints to the CMS or state agencies on behalf of the CMS.

### The Joint Commission

Since 1961, review of blood use has been an element of the accreditation process of The Joint Commission.<sup>27</sup> By 1970, The Joint Commission required review not only of blood utilization, but of transfusion reactions as well. The 1986 standards were more comprehensive, mandating review of transfusion policies and procedures, ordering practices, and adequacy of the transfusion service generally. The standards also required evaluation of all transfusions.<sup>99</sup> After repeated blood utilization review consistently documented appropriate blood use, sampling became acceptable. Although only a minority of hospitals complied with this requirement for 100% evaluation, this standard was nevertheless the driving force behind blood utilization review in the United States.

Since 1991, The Joint Commission has made a series of modifications to this standard, and 100% review is no longer required. The actual number of transfusions to be reviewed is not mandated, although recommendations are provided. Additionally, as part of an effort to reduce medical errors, The Joint Commission has developed National Patient Safety Goals; the number one goal is to “improve the accuracy of patient identification.” Although this safety goal was implemented because of the recognition that patient misidentification affects all aspects of medical care, its relevance to transfusion therapy was clearly recognized by The Joint Commission. One of the implementation expectations for the goal clearly states that “Two patient identifiers are used when administering medications or blood products” and “when collecting blood samples ...for clinical testing.” There are also explicit statements that specimens collected from a patient must be labeled at the bedside and that the patient’s location may not be used as an identifier. The patient safety goal is reiterated in the body of the standards themselves. An element of performance of standard PC 5.10 also requires two identifiers for patient samples.

The Joint Commission views transfusion services from a dual perspective. Standards are devoted both to the entire process of blood transfusion from blood ordering through infusion and to the laboratory procedures and practices. Two accreditation manuals have standards regarding blood transfusion. The *Comprehensive Accreditation Manual for Hospitals*<sup>100</sup> provides specific standards for hospitals that transfuse and monitor

blood components. The *Comprehensive Accreditation Manual for Pathology and Clinical Laboratory Services*<sup>101</sup> contains technical standards that are patterned after the CLIA 1988 requirements and AABB standards. This dual approach provides The Joint Commission with the opportunity to assess the entire spectrum of clinical and laboratory blood transfusion practices. The Joint Commission Laboratory Accreditation Program has deemed status with the CMS.

Standards related to blood transfusion are included in the *Comprehensive Accreditation Manual for Hospitals* in those sections addressing the medical staff, provision of care, treatment and services, management of information, improvement of organization performance, environment of care, and sentinel event review. Among the salient standards, MS 3.10 states that the medical staff must have “a leadership role in hospital performance improvement activities to improve quality of care.” One of the specific elements of performance for MS 3.10 requires that the medical staff be “actively involved in measurement, assessment and improvement in the use of blood and blood components.” A similar provision, PI 1.10 in the section on performance improvement, states that the hospital must “collect data to monitor its performance,” and this requirement includes collecting data on blood and blood product use. PI 2.20 requires that “undesirable patterns and trends in performance are analyzed,” including all confirmed transfusion reactions. In addition, hemolytic transfusion reactions involving the administration of blood having major blood group incompatibilities are identified as reviewable sentinel events subject to specific review by The Joint Commission.

The *Comprehensive Manual for Laboratory and Point of Care Testing*<sup>10</sup> contains more specific provisions governing transfusion services. HR 1.15 requires that the director of the blood bank must be a physician, either a pathologist or other physician qualified in immunohematology and hemotherapy. Sections QC 5.10 through QC 5.260 provide guidance on what The Joint Commission regards as critical elements comprising an acceptable transfusion service. Section QC 5.10 requires that the transfusion service have written policies and procedures that “are acceptable in format, content, review process and availability.” These policies and procedures must be consistent with AABB standards, and must be reviewed annually. There must also be policies and procedures governing transfusion reactions and adverse events. Every adverse event must be evaluated by the medical director and documented in the patient’s medical record.

### College of American Pathologists

The CAP laboratory accreditation program expects a participant laboratory to demonstrate that it is in compliance with the CAP standards for laboratory accreditation. These standards relate to requirements for laboratory direction, physical facilities and safety, quality control and performance improvement, and inspection. Assessment of whether a laboratory meets the standards is accomplished through a series of checklists.

Any applicable question that cannot be answered “yes” is considered a deficiency and must be corrected within 30 days with the submission of supporting documentation for accreditation to be achieved. The inspector does not grant or deny accreditation, but makes a recommendation. The accreditation decision is made by the CAP Commission on Laboratory Accreditation. In addition to the on-site inspection program, the CAP laboratory accreditation program monitors proficiency testing performance of its participant laboratories. The CAP has deemed status with The Joint Commission and the CMS.

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## Transfusion Medicine Committee

A clinical staff committee concerned exclusively with practices and policies related to transfusion and the transfusion service is not mandated by The Joint Commission or other accreditation entities. However, it has become routine in most hospitals because a standing transfusion committee is an efficient way of meeting quality assurance and peer review requirements.<sup>102</sup> It reports to the health-care evaluation office or committee, or directly to the medical policy committee of the institution.

### Membership

The membership of the transfusion committee should represent the departments that are the principal users of blood and blood components, such as hematology or oncology, surgery, and anesthesiology. Obstetrics/gynecology and pediatrics departments also should be represented if these departments are part of the hospital. In addition, staff from some of the surgical subspecialties (eg, cardiovascular surgery) and from services with specialized needs (eg, neonatal, emergency, intensive care) are appropriate members. The transfusion service supervisor is a valuable resource concerning laboratory operations and the feasibility of proposed policies. Clinical staff members with special interests or expertise in transfusion medicine should be encouraged to accept appointment on the committee. Committee activities are educational for the participating physicians.<sup>103-105</sup> Transfusion committee appointments should last several years so that expertise can be acquired and should be staggered to ensure continuity.

The medical director of the transfusion service must be a committee member; however, it is preferable for the director not to serve as chair of the transfusion committee. Changes in policy are more readily accepted if the transfusion committee is perceived as a distinct entity, independent of the transfusion service. As the expert, the director always has the opportunity to present views and shape policy. However, having a consumer of blood products serve as chair is likely to enhance the credibility, visibility, and effectiveness of the transfusion committee.

Other services in the hospital that often send representatives to the transfusion committee meetings include the hospital

compliance office; the divisions of quality, risk, and utilization management of the health-care evaluation office; the medical records department; medical and surgical nursing services; and the intravenous therapy team. If the hospital has a residency training program, it is helpful to have a resident physician present for committee meetings. The director of the blood center that supplies the hospital may be a valuable resource to provide comparisons of transfusion activities at different hospitals in the community, advice on meeting regulatory requirements, and education on transfusion-related issues.<sup>106</sup> For example, if one hospital follows markedly different policies and does not encounter differences in outcome, this information can be the basis for adopting an alternative approach. When policies with medicolegal implications are under discussion (eg, a policy for recipient consent for blood transfusion, implementation of look-back procedures), it is useful to have the hospital attorney attend committee meetings.

A multidisciplinary transfusion committee can serve as an active and effective guiding force in the development of policies and procedures related to the transfusion of blood components. Physicians must give sufficient priority to this process, and the hospital administration must make the necessary resources available to ensure effective committee work. A prompt and thoughtful response to committee recommendations by hospital and clinical governing bodies is essential to creating the correct environment. In academic centers, department chairs should support the participation of faculty on clinical staff committees and appreciate that this is an important element of the faculty member's educational and service efforts. Data derived from the committee's work can serve as a source of clinical scholarship.

### Functions of a Transfusion Committee

The transfusion committee should be involved in evaluation of all aspects of transfusion and transfusion practice in the hospital. Although utilization review is perhaps the most well known and obvious function of the transfusion committee, the committee should also be the leader in establishing hospital-wide policies and procedures governing transfusion practice as well as sponsoring educational activities to improve transfusion practice in the hospital.<sup>107</sup> Each transfusion committee should determine the topics it wants to consider. It should select areas that will help the institution achieve compliance with accreditation standards and regulatory requirements and focus on those most in need of systematic oversight or improvement. High-volume and high-risk procedures have been suggested as important focal points for review of transfusion practices.<sup>108</sup>

### Utilization Review

Although effective utilization review need not involve the transfusion committee, the participation of the committee supplements other efforts. The functions can also be performed by transfusion service physicians prospectively or soon after transfusion. The transfusion committee and others performing utilization review should be familiar with state laws and regulations

governing the discoverability for legal proceedings of the committee's minutes, records, correspondence, and reports. The committee and others need to shape their practices accordingly. State statutes often prevent the routine release of these documents as a result of legal discovery proceedings, but sometimes permit courts to order the disclosure of such records if it is found that extraordinary circumstances make such an action desirable. Considerable caution must be taken regarding the content of all written records with respect to their discoverability.

### Undertransfusion

A patient who needs a blood transfusion but does not receive one is more likely to be harmed than is a patient who receives a transfusion that is unnecessary. Most attention has been devoted to identifying unnecessary transfusions. However, hospital transfusion committees may want to determine whether patients who need transfusions are receiving them.<sup>109</sup> For example, the members of the transfusion committee may want to review the medical records of patients with hemoglobin levels less than 5.5 or 5.0 g/dL who did not receive transfusions.

There is great variability in transfusion practices among institutions. This variability illustrates that good clinical data are often lacking and indications for transfusion are not universally accepted. This prompts concern that undertransfusion could be a problem. Some data suggest higher hematocrit levels are needed to prevent myocardial ischemia after surgery in selected patients.<sup>110</sup>

Undertransfusion can also occur if units of blood are not being released from the blood bank in a sufficiently timely manner.<sup>111</sup> The publicity regarding the risks of transfusion and inappropriate transfusions and the strict policies, procedures, accreditation standards, and regulations governing blood banks may have combined to create undue concern about the release of blood components for transfusion. Members of transfusion committees may want to ensure that blood bank technical staffs are not delaying the release of urgently required components because of excessive concern about exceptions to routine practice.

### Unnecessary Transfusion

Review of transfusion practices has the potential to improve the quality of clinical care, educate clinical staff, reduce costs, ensure regulatory and accreditation compliance, reduce the risk of litigation, provide information about the practices of individual physicians and specific clinical services, and establish consistent practices. It is onerous and unnecessary to review intensively each transfusion in an institution. Rather, the transfusion service director and medical staff representatives of each hospital should determine the timing, scope, and intensity of the review. One approach used by many hospitals is to review only orders for transfusions for which certain predetermined screening criteria are not met. Intensive assessment of an individual case also may be initiated in response to concerns of a patient, provider, or third-party payer.

### Timing of Utilization Review

Utilization review can be conducted prospectively or retrospectively.<sup>112</sup> Prospective review requires verification by a reviewer that criteria have been met before a blood component is made available for transfusion. Although this on-site validation is desirable and presumably the most beneficial, it is labor intensive and not always feasible in an emergency. Furthermore, in the care of an acutely ill patient, a discussion regarding transfusion practice is not conducive to effective education and is sometimes inappropriate. Although prospective auditing may prove useful for customized blood products, such as irradiated components or unusually large orders, it is not likely to be feasible for all blood transfusions within an institution. Retrospective audits are most effective if performed soon after the transfusion—for example, within 24 hours. Although common sense suggests that this prompt feedback would be useful to physicians, it requires a continuing effort and is not always practical. Retrospective audits later are the least difficult to perform but also should be completed in as timely a manner as possible to be maximally effective. There are no data to substantiate the effectiveness of retrospective review alone in improving transfusion practices, although there are many reports of success with other approaches, some of which have been combined with retrospective review.

One method that has been used frequently requires prospective justification with either a computer-generated ordering menu or simply a requisition slip. In both cases, screening criteria are used as part of the ordering process. The ordering physician selects from a menu the transfusion criterion met by a particular patient or presents another clinical justification for transfusion. Table 66-2 contains examples of screening criteria that are used. Screening criteria for adult<sup>114,115</sup> and pediatric<sup>116,117</sup> blood transfusion practices have been published. All or some of the orders in which another reason was provided as the indication for transfusion are reviewed more intensively, either before or after the transfusion. Transfusions thought to pose the greatest risk or be the most likely to be inappropriate can be reviewed. For hospitals that do not use screening criteria, these unusual transfusion episodes are a logical place to begin blood utilization review. Each type of component transfused in the institution should be reviewed, and each clinical service that uses blood should be evaluated.

If a retrospective review is conducted long after the transfusion, the reviewer must consider the information available to the clinician and the patient's clinical condition at the time the decision to perform transfusion was made.

### Screening Criteria

Criteria used for evaluating clinical care should ideally be established from a scientific database. However, this information is not always available, and it may be necessary to use a consensus opinion.<sup>118</sup> Implicit criteria are used when a designated expert reviews a particular case and judges the quality of care. Explicit criteria are specified in advance, before the assessment of individual cases is undertaken. Reviews based on implicit criteria can be individualized but are costly and inherently imprecise.<sup>119,120</sup>

Explicit criteria can be used to make reproducible assessments at a relatively low cost. They are, however, not responsive to the subtle variations in individual patients that can prompt different standards of therapeutic intervention. Implicit and explicit criteria frequently are combined in a particular quality assessment program of clinical practices. Explicit criteria often are used to separate cases that should not require further review (because they most likely have already met accepted standards of practice) from those that need to be reviewed individually (according to implicit criteria or a combination of implicit and explicit criteria). As institutions measure and assess various processes, criteria have to be modified as new clinical information becomes available and a different level of performance is required.

Although explicit screening criteria are useful to select transfusion episodes for intensive review, the criteria do not constitute a standard of practice. Not every patient who meets a screening criterion necessarily needs a blood transfusion, and there are patients who do not meet the screening criteria who need a transfusion. It is necessary to differentiate between criteria used simply to select a medical record for further intensive review and what is appropriate clinical practice. Nevertheless, those who control the screening criteria exert great influence on clinical practice. Therefore, the committee might want to review selectively some cases in which a transfusion has fallen within the criteria. Initially the criteria can be designed liberally to capture transfusion episodes in which inappropriate practices were most likely to have occurred. However, once these have been addressed, the criteria can be modified to become more restrictive. Differences in patient populations, professional judgment, and prevailing practices result in different screening criteria among hospitals. The clinical staff has overall responsibility for approving the screening criteria. The intriguing suggestion has been made that hospitals' clinical staffs should review each other to avoid the collegiality that arises within institutions.<sup>121</sup>

The input and approval of practicing physicians are helpful to lessen the debate about the validity of screening criteria. The criteria should be limited to one or a few easily identified elements, and exceptions should be minimized. Policies regarding the administrative response to deficient transfusion practice should be publicized, so that there will be no surprises when blood utilization review is implemented. The screening criteria should be publicized regularly. If the criteria are not on an order menu, house staff and new members of the clinical staff should receive copies of the criteria, which should be disseminated throughout the institution.

Screening criteria should be evaluated on a regular basis. New clinical practices require reassessment of the criteria. Intensive assessment of a medical record or subsequent discussion about a specific clinical practice with a treating physician may prompt a revision of the criteria.

An alternative approach is a system in which the admission and discharge hematocrits of patients are used to determine the appropriateness of blood transfusions.<sup>122,123</sup> The advantages of this system include possible elimination of extensive chart audits, individualization of reviews according to patient or procedure,

## Section VI

**Table 66-2.** Threshold Criteria for Blood Utilization Review\*

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### Red Blood Cells

- Hematocrit less than 25%
- Acute blood loss greater than 20% of blood volume
- Coronary or cerebral vascular disease and hematocrit less than 30%
- More than 10% surface burns and hematocrit less than 30%
- For neonates: hematocrit less than 40% with respiratory distress; otherwise, hematocrit less than 30% or blood loss more than 10% of blood volume

### Platelets

- Bleeding or a planned invasive or surgical procedure and any of the following:
  - Platelet count  $<80,000/\mu\text{L}$
  - Bleeding time longer than 7.5 minutes
  - Documented platelet function disorder
  - Administration of more than one blood volume of red cells and other volume-expanding fluids in previous 24 hours
- Platelet count  $<100,000/\mu\text{L}$  in a patient who is either of the following:
  - Experiencing retinal or cerebral hemorrhage
  - Recovering from a cardiopulmonary bypass procedure
- Prophylactically for platelet count  $<20,000/\mu\text{L}$  ( $<40,000/\mu\text{L}$  in neonate)
- Neonate with bleeding or a planned invasive or surgical procedure and platelet count  $<100,000/\mu\text{L}$

### Fresh Frozen Plasma

- Hemorrhage or a planned invasive or surgical procedure and either of the following:
  - International normalized ratio 1.5 or higher (other than for central nervous system)
  - International normalized ratio type 1.3 or higher (for central nervous system)
- Documented deficiency of Factor II, V, VII, X, or XI
- Administration of more than one blood volume of red cells and other volume-expanding fluids in previous 24 hours
- Thrombotic thrombocytopenic purpura
- Hemolytic uremic syndrome

### Cryoprecipitate

- Bleeding or a planned invasive or surgical procedure and any of the following:
  - Documented deficiency of fibrinogen ( $<80\text{ mg/dL}$ )
  - Documented von Willebrand disease
  - Documented deficiency of Factor XIII
  - Uremic platelet dysfunction (bleeding time longer than 7.5 minutes)

### Plasma Cryoprecipitate Reduced

- Bleeding or a planned invasive or surgical procedure and documented deficiency of Factor II, V, VII, X, or XI
- Thrombotic thrombocytopenic purpura
- Hemolytic uremic syndrome

### Granulocytes

- Neutropenia ( $<1000/\mu\text{L}$ ) with documented infection unresponsive to antibiotics

### Rh Immune Globulin

- Postpartum Rh-negative mother with Rh-positive infant
  - Antepartum Rh-negative woman at 28 weeks of gestation or with risk of recent fetomaternal hemorrhage or who received antepartum Rh Immune Globulin 12 weeks previously
  - Rh-negative woman after abortion, ectopic pregnancy, miscarriage, or amniocentesis
  - Transfusion of Rh-positive cellular blood components to Rh-negative patient
  - These are not indications for transfusion. They are criteria that may be used to preclude more intensive review of blood transfusion. They may be incorporated into blood ordering forms or computer programs. Adapted with permission from Mintz.<sup>113</sup>
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and lack of dependence on a single hematocrit level at either admission or discharge, which can be misleading.

### *Consequences of Utilization Review*

A retrospective utilization review must consider carefully the clinical situation at the time the transfusion was given. If, however, it

is determined that a transfusion deviated from acceptable practice, the involved physician should be informed in writing and invited to provide an explanation for the transfusion in question. After the physician's response is received, the committee makes a final determination regarding the transfusion and informs the physician. Audit findings should be summarized for departments

and divisions and be made available to their chairs and directors. If blood transfusion practices are audited selectively, the review should be rotated so that at some point all of the divisions within the hospital are subject to review. Collected information should be sent regularly to the committee or individual to whom the transfusion committee reports. When particular problems with transfusion practices are identified, they should be subjected to repeated intense evaluation. The overall effect of the review program on clinical practices may be assessed as well.

It is important to communicate conclusions regarding appropriate transfusion practices to the clinical staff. These communications can reinforce good practices and increase the openness of practitioners to future, perhaps less supportive comments. Because an important element of any quality assessment program is the continuing education of the clinical staff, the auditing process should be designed more as a tutorial activity than as a punitive one. Improperly performed audits can antagonize those whom they are designed to instruct, thereby hindering acceptance of other well-designed programs. If the institutional leadership provides the necessary resources for the auditing process and physicians provide sufficient time and effort, utilization review can be an important element in documenting sustained excellence or continuing improvement in clinical practices.

### **Oversight of Transfusion Policies, Procedures, and Guidelines**

The transfusion committee should provide oversight of hospital-wide policies and procedures. It should also oversee the entire transfusion process, carefully examining each step in the transfusion chain to ensure compliance with safety concerns, regulatory requirements, and clinical appropriateness.<sup>124</sup> It is very important that transfusion policies be consistent throughout the hospital. Consistency encourages compliance with transfusion procedures and facilitates staff training. The transfusion committee plays an important role in ensuring this consistency. Table 66-3 and the list below identify selected items regarding the overall transfusion process that either the transfusion service or the committee may wish to monitor.

1. Review the institution's policies and procedures for proper patient, specimen, and blood component identification and other policies and procedures related to blood administration.
2. Review that transfusions are appropriately documented in the medical record. This activity should include a review of the documentation required by the institution's policies. The blood transfusion record can be audited to ensure that it includes the information the institution requires regarding the intended recipient's name, identification number, and ABO group and Rh type; the component's unique identification number; the donor's ABO group and Rh type; interpretation of compatibility tests; the volume administered; the expiration date or time of the component; time of transfusion; identification of the transfusionist; and information about patient monitoring during the transfusion. A systematic education program for transfusion nurses can improve documentation and monitoring procedures.<sup>126</sup> The review can include verification that

the medical record contains the documentation of the indications for transfusion, the prescription for the transfusion, and the outcome of the transfusion.

3. Review compliance with recipient consent process for blood transfusion. This includes determination that consent has been obtained, that the required signatures (patient, witness, and physician) are included, and that the consent is appropriately dated and placed in the record.
4. Review that there is appropriate documentation in the medical record of prescription for transfusion, indications for transfusion, and outcome of transfusion.
5. Ensure that adequate policies and procedures exist for detecting, reporting, and evaluating transfusion reactions, and review the timeliness and adequacy of the investigations of these adverse events. Assess whether reasonable effort is made to ensure the reporting and evaluation of suspected instances of transfusion-transmitted infectious disease.<sup>127</sup>
6. Review the hospital's look-back activities to ensure that they comply with federal regulations and accreditation standards and are being completed on a timely basis.
7. Evaluate the utilization of devices used in blood transfusion. The committee can ascertain that appropriate quality control has been performed on blood warmers and infusion pumps, the devices used are appropriate for the purpose, and personnel have been trained adequately in use of devices.
8. Review autologous blood collection and transfusion activities. A quality improvement team significantly improved the availability of autologous donor blood and had a far-reaching effect on quality improvement methods throughout one medical center.<sup>128</sup> The committee can review how such services are being used, whether allogeneic blood was ever transfused when autologous blood was available, and whether autologous blood has been transfused more liberally than the committee deems appropriate. In view of the risks of bacterial contamination, clerical error, and hypervolemia that exist with autologous blood transfusion, the committee can reasonably require that the same standards used for allogeneic blood transfusion be used for autologous blood transfusion. An alternative, because autologous blood is safer, is for the transfusion committee to accept more liberal indications for transfusion of autologous blood. In either case, the same screening criteria can be used to determine which cases require more intensive review. This practice allows individual consideration of the use of autologous blood in situations in which allogeneic transfusion would be considered inappropriate. The collection, manipulation, and utilization of autologous blood collected intraoperatively, postoperatively, and after trauma also can be reviewed.
9. Review blood bank performance in such areas as timeliness of response to requests, productivity, and outdating of products.
10. Review the utilization of certain customized products such as irradiated blood.
11. Review the use and underuse of single-unit transfusions. Single-unit transfusion sometimes represents good practice. If one RBC transfusion increases oxygen delivery adequately, there is no need for additional transfusions.



**Table 66-3.** Factors to Consider in Monitoring the Ordering, Dispensing, and Administration of Blood Components

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|     |                                                                                                                                     |
|-----|-------------------------------------------------------------------------------------------------------------------------------------|
| 1.  | What time was request for blood component received?                                                                                 |
| 2.  | Was there a prescription for the transfusion?                                                                                       |
| 3.  | Was the request submitted correctly?                                                                                                |
| 4.  | Was the correct component selected for issue?                                                                                       |
| 5.  | Was the component inspected?                                                                                                        |
| 6.  | Was the component verified to be in date?                                                                                           |
| 7.  | Was documentation before issue completed correctly?                                                                                 |
| 8.  | What time was the transportation department notified that the component was available?                                              |
| 9.  | What time was the component issued?                                                                                                 |
| 10. | Was the correct filter issued?                                                                                                      |
| 11. | Was documentation regarding transportation completed correctly?                                                                     |
| 12. | What time was the component delivered by transportation?                                                                            |
| 13. | Was the blood component unattended after release from the blood bank? Where was it?                                                 |
| 14. | Was informed consent obtained in accord with written procedure?                                                                     |
| 15. | Were patient and blood component identification properly performed in accord with written procedure before transfusion was started? |
| 16. | Was documentation on the transfusion slip accurate and complete?                                                                    |
| 17. | Were pretransfusion vital signs obtained and recorded?                                                                              |
| 18. | Was the filter attached properly?                                                                                                   |
| 19. | Was arm/port preparation properly performed?                                                                                        |
| 20. | When was the transfusion started? Was this documented?                                                                              |
| 21. | Were only approved solutions in contact with the component?                                                                         |
| 22. | Were only approved solutions mixed with the component?                                                                              |
| 23. | If utilized, was a blood warmer or infusion pump used in accord with written procedure?                                             |
| 24. | Were vital signs obtained and recorded during the transfusion in accord with written procedure?                                     |
| 25. | Was any transfusion reaction reported to a physician and the blood bank in accord with written procedure?                           |
| 26. | What time was the transfusion completed? Was this documented?                                                                       |
| 27. | Were vital signs taken and recorded, and was the patient observed after transfusion in accord with written procedures?              |
| 28. | Were the component bag and other materials disposed of correctly?                                                                   |
| 29. | Were written instructions about adverse reactions provided if the recipient was an outpatient or at home?                           |
| 30. | Was all documentation placed in the patient's permanent record in accord with written procedure?                                    |
| 31. | Was the outcome of the transfusion documented in the medical record?                                                                |

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Used with permission from Mintz.<sup>125</sup>

12. Ensure that the last unit or units of several transfusions given sequentially are appropriate. A single low hemoglobin concentration or episode of bleeding can lead to the transfusion of several units of blood, of which the last or last few units may have been the least needed transfusions provided. These transfusions may not come routinely to the attention of the committee because the patient may have met a particular criterion initially. The committee may want to assure itself that when more than one unit of blood is transfused for a specific criterion, each of the units is truly indicated. In one study, 2-unit transfusions were commonly given to patients undergoing total hip replacement when 1 unit would have sufficed.<sup>129</sup> In another study, intertransfusion assessment was frequently not completed for

postoperative patients.<sup>130</sup> Results of another study suggested that determining the hematocrit immediately before administration of each unit of blood would have reduced by 25% the amount of blood transfused to patients undergoing colonic resection.<sup>131</sup>

13. Review whether the institution's guidelines for preoperative ordering of blood crossmatching<sup>5</sup> are followed. The effective use of preoperative crossmatching guidelines for elective surgery has been shown not to compromise patient care.<sup>132</sup>

14. Review the veracity of the ordering physicians in choosing the criteria met to justify blood transfusion, if the institution has a system in which physicians indicate in advance the reason for the transfusion.

15. Review blood wastage caused by failure to return unused units to the blood bank on a timely basis.<sup>133</sup>

16. Audit the blood bank staff to ensure that they are following written policies and procedures for handling and release of blood from the blood bank. Audit the procedures to determine whether appropriate review has occurred within the past 12 months.

17. Audit the transfusion practices on various clinical services to ensure that staff appropriately identify patients before transfusion, appropriately identify blood components before administration, appropriately monitor the patient during transfusion, and appropriately document the transfusion episode in the patient's hospital record. Boone et al<sup>134</sup> found that careful monitoring can effectively identify correctable errors in blood administration.

18. Review the timeliness and adequacy of delivery of blood components from the blood supplier. It may be helpful to invite blood center management to attend discussions regarding this issue.

19. Review blood bank proficiency testing, accreditation reports, and quality control results.

20. Determine, on occasion, the crossmatched to transfused ratio from selected services or within the entire institution. Although this calculation was emphasized in the past, its utility has frequently been overstated. There is no accreditation requirement to determine this statistic. However, institutions or selected departments or divisions that show a significantly elevated ratio, might be ordering more blood than is reasonable. An appropriate ratio is less than 2.

21. Assist in the development of hospital policies related to transfusion practices. In one study, significant decreases in red cell usage during four common surgical procedures were seen after the hospital transfusion committee introduced a requirement that hemoglobin be less than 8g/dL in asymptomatic patients.<sup>135</sup> Members of the transfusion committee could also help to shape policies regarding recipient consent for blood transfusion, the availability of directed donations from friends or relatives of patients, and the development of look-back procedures for patients who may have received blood from donors subsequently known to have infection with an agent transmissible through blood transfusion.

22. Serve as an advocate for the blood bank to the hospital's clinical staff and administration for personnel, equipment, or space needs.

23. Review staff training and continuing education in blood administration to ensure competence of personnel who transfuse blood components.

## Education

The transfusion committee stands in a unique position to advocate optimal transfusion practice. Although the utilization review process itself provides an opportunity for education of individual clinicians about their ordering practices and use of blood products, other modalities are available to the committee. A hospital

intranet affords the committee a venue for posting important transfusion-related information. Such important items as appropriate transfusion criteria, surgical crossmatch ordering guidelines, signs and symptoms of adverse reactions to transfusion and transfusion-related policies can be posted for easy referral by hospital staff. A web site is also easily updated as new medical knowledge or new guidelines in transfusion medicine become available. Each individual committee member is also an important educational resource. Transfusion committee members can take important information back to their respective departments. They can also provide one-on-one informal teaching to residents and hospital staff when transfusion-related issues come up during daily practice.

## Summary

Although blood components typically represent only approximately 1% of an average hospital's budget, transfusion is at once a life-saving and potentially life-threatening procedure. Remarkable progress has been made in reducing the transmission of viruses through blood transfusion, but this advance has not been matched in improved safety with respect to other important transfusion hazards. Although knowledge is still incomplete regarding indications for blood component transfusions, quality assessment programs for blood transfusion are needed to improve transfusion practice.

A successful quality program creates "an environment of watchful concern"<sup>26</sup> and is conducted in a professional, nonadversarial, and educational manner. Assessment of transfusion practices has the potential to enhance the knowledge and judgment of health-care professionals, provide significant information about patient care, reduce the risk of litigation, decrease costs, ensure compliance with regulatory and accreditation requirements, conserve the blood supply, provide an opportunity to demonstrate quality and value to the public, and above all help create, sustain, and document excellence in patient care.

## Disclaimer

P. Clark has disclosed no conflicts of interest. P. Mintz has disclosed financial relationships with Amgen Navigant, Cerus, Fenwal, Immucor and CL Behring.

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