Artificial Blood

Jerry E. Squires

Blood substitutes are under development for transfusion in place of donor blood during emergencies and lengthy surgeries. The first generation of blood substitutes is currently in clinical trials.

The idea of using a blood substitute instead of human blood for transfusing patients is not new. In the 17th century, Sir Christopher Wren suggested that ale, wine, and even opium could be used as substitutes for human blood. It was not until 1901 that the modern age of blood transfusion began with the discovery of human blood group antigens by Karl Landsteiner (1, 2). He categorized human blood into A, B, and C (later renamed O) groups, and a year later the AB group was added to the list. These studies were critical to understanding why blood transfusions had failed thus far. Ottenberg in 1913 was the first to apply blood group serology to transfusion practice. Even with Ottenberg's description of blood compatibility testing, blood transfusions were severely limited because of a lack of suitable anticoagulants and storage methods. The First and Second World Wars precipitated progress on these two fronts, at last allowing blood transfusion to become a standard part of medical treatment.

One of the principal tasks of blood is to transport oxygen throughout the body and then to release the oxygen to tissues, picking up carbon dioxide in its stead (1, 2). All of this is accomplished by hemoglobin, the oxygen-carrying protein contained within red blood cells (erythrocytes). The other cellular constituents of blood are white cells, which are important for the immune response, and cellular fragments called platelets that are crucial for blood clotting and wound healing.

Although transfusion of donor blood is a routine and safe procedure, there are several

American Red Cross Biomedical Services, Arlington, VA 22307, USA. E-mail: squiresj@usa.redcross.org

reasons for developing blood substitutes. Human red blood cells have exacting storage requirements designed to prolong clinical effectiveness and to reduce the risk of bacterial contamination. This substantially limits the availability of blood at disaster sites or on the battlefield. Blood substitutes with less stringent storage requirements would be valuable in these situations. In addition, blood substitutes are more amenable to sterilization to remove infectious pathogens, and they do not require cross-matching because they do not harbor blood group antigens. Donor blood shortages make blood substitutes attractive for the short-term replacement of erythrocytes during surgery. In the 1980s, the realization that the human immunodeficiency virus (HIV) could be transmitted through blood transfusion provided renewed impetus for the development of "disease-free" blood substitutes. Before the development of a specific test for HIV, the risk of transfusion-associated acquired immunodeficiency syndrome was about 38 per 100,000 transfused patients.

Researchers interested in developing blood substitutes (dubbed artificial blood) have predominantly concentrated on mimicking the oxygen-carrying capacity of hemoglobin. However, in addition to being able to transport oxygen, an ideal blood substitute would also (i) require no cross-matching or compatibility testing, (ii) be suitable for long-term storage (preferably at room temperature), (iii) be able to survive in the circulation for several weeks (the intravascular "dwell" time) before being cleared by the kidney, (iv) be free of side effects, (v) be free of pathogens, and (vi) not only transport but also effectively deliver oxygen to tissues. There

are two types of blood substitute currently under development: those derived from hemoglobin, called hemoglobin-based oxygen carriers (HBOCs), and those that use perfluorocarbon emulsions (3).

## Hemoglobin-Based Oxygen Carriers

The hemoglobin in adult erythrocytes is a tetramer of two α and two β polypeptide chains, each of which is bound to an iron-containing heme group. Each heme group binds one oxygen molecule. This oxygen-heme bond results in a conformational (shape) change in the hemoglobin molecule, which in turn progressively increases the affinity of hemoglobin for additional oxygen molecules. Thus, a small change in oxygen partial pressure results in a large change in the amount of oxygen bound or released by hemoglobin (Fig. 1). A number of additional factors, such as temperature and pH, can alter the oxygen-hemoglobin dissociation curve (Fig. 1). Similarly, 2,3-diphosphoglycerate (2,3-DPG), a product of the red blood cell glycolytic pathway and present in normal human erythrocytes, also directly affects the binding of oxygen to hemoglobin. As the concentration of 2,3-DPG rises, the oxygen-hemoglobin dissociation curve "shifts to the right," thereby allowing the release of oxygen to tissues at higher than normal oxygen partial pressures (Fig. 1).

A cell-free solution of hemoglobin can be used as a blood substitute because hemoglobin maintains its ability to transport oxygen outside of the red blood cell. One advantage of HBOCs is that compatibility testing is not required. In contrast, transfusion of donor blood requires careful cross-matching to avoid the consequences of a hemolytic transfusion reaction in the recipient, the cause of a small but significant transfusion-associated morbidity and mortality. Another advantage of cell-free hemoglobin is that it can be sterilized by ultrafiltration and low heat to inactivate infectious agents, a strategy that is not possible with red blood cells.

Early attempts to develop blood substitutes in the 1970s concentrated on cell-free solutions of human hemoglobin (4). But problems with these HBOCs included unacceptably short survival times in the circulation, an abnormally high oxygen affinity, and clinical side effects such as malaise, abdominal pain, hemoglobinuria, and renal toxicity. If HBOCs are to become clinically effective blood substitutes, two

Fig. 1. Oxygen-hemoglobin dissociation curves. A small change in oxygen partial pressure results in a large change in the amount of oxygen released or bound by native hemoglobin. Under conditions of decreased pH, increased temperature, or an increase in the concentration of 2,3-DPG, the oxygen-hemoglobin dissociation curve is shifted to the right, thereby enabling the release of oxygen to tissues at higher than normal oxygen partial pressures. An increase in pH or a decrease in temperature or 2,3-DPG concentration shifts the oxygen-hemoglobin dissociation curve to the left, resulting in an increased affinity of hemoglobin for oxygen.
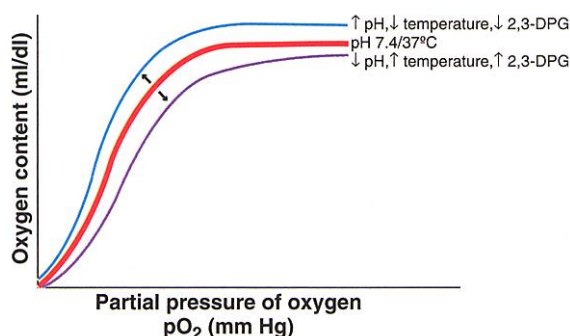
principal challenges must be overcome: their short intravascular dwell times, and their reduced ability to oxygenate tissues relative to normal hemoglobin.

The first challenge is to prevent the rapid breakdown and elimination by the kidney of hemoglobin molecules that are no longer within the protective environment of the red blood cell. Intracellular hemoglobin has the same life-span as the erythrocyte, about 120 days, but in solution the hemoglobin tetramer readily dissociates into monomers and dimers that are quickly eliminated by the kidneys. The dissociation of oxygen from intracellular hemoglobin is modified by 2,3-DPG, resulting in a normal P_{50} value (the oxygen partial pressure at which hemoglobin is 50% saturated) of about 27 mmHg for human blood. In hemoglobin solutions, the lack of 2,3-DPG causes a "left shift" in the oxygen dissociation curve, that is, an increase in the affinity of hemoglobin for oxygen. Although this increased affinity does not limit the ability of HBOCs to transport oxygen, it does seriously limit their ability to "unload" or deliver oxygen to tissues.

These two challenges have been addressed by chemically modifying cell-free hemoglobin. In the first class of modified HBOCs, specific chemical cross-links are established between hemoglobin polypeptide chains to prevent the dissociation of the hemoglobin tetramer, thus retarding renal elimination. Treatment with 3,5-dibromosalicyl fumarate results in a strong covalent bond that maintains the integrity of the hemoglobin tetramer (5, 6). This results in a prolonged intravascular dwell time of up to 12 hours; by contrast, untreated cell-free hemoglobin is eliminated by the kidney in less than 6 hours. Alternatively, hemoglobin can be treated with bifunctional cross-linking agents, such as o-raffinose or glutaraldehyde, that target specific amino groups and polymerize the hemoglobin molecule (5, 6). These reagents produce polyhemoglobin, composed of four or five hemoglobin molecules, which varies in molecular size and configuration and has intravascular dwell times of up to 24 hours. Finally, hemoglobin can be conjugated to a variety of larger molecules such as dextran, polyethylene glycol,

or polyoxyethylene, retarding the rate at which it is cleared from the circulation. In this instance, the intravascular dwell time can be extended to 48 hours (5). New HBOCs under development include polyhemoglobin linked to antioxidant enzymes such as superoxide dismutase and catalase in an effort to minimize ischemia-reperfusion injury (6). In addition, some groups are trying to package human hemoglobin in artificial red blood cells made from biodegradable polymer nanocapsules or lipid vesicles (6).

There are at least three modified hemoglobin products currently in advanced clinical trials (see the News story by Stokstad, page 1003). Northfield Laboratories has developed Polyheme, a polymerized human hemoglobin product. Biopure has prepared polymerized hemoglobin from bovine red blood cells (Hemopure), which has received approval for use as a blood substitute in the Republic of South Africa and is now awaiting FDA review of a phase III clinical trial in the United States. Finally, Hemosol has developed Hemolink, a partially polymerized human hemoglobin product that is also under FDA review.

There are several problems with HBOCs that must be overcome. The first is the source of the hemoglobin used in the blood substitute. An obvious candidate is human hemoglobin derived from outdated donor blood (red blood cells that have exceeded the approved storage period), but the limited supply of human blood has compounded difficulties in developing a human-derived HBOC. A more readily available (and cheaper) source of hemoglobin is bovine blood. Bovine hemoglobin does not have 2,3-DPG, resulting in a P_{50} of about 30 mmHg both inside and outside the red blood cell (similar to that of the intracellular native human hemoglobin). In addition, once stripped of all other proteins, bovine hemoglobin is not recognized by the human immune system as foreign. However, one potential obstacle to the acceptance of a bovine-derived HBOC is the fear that it could harbor the prion pathogen that causes bovine spongiform encephalopathy. Genetically engineering bacteria to produce a recombinant source of human hemoglobin would eliminate the concerns about disease transmission associated with hemoglobin

derived from humans or animals. Of course, the problem with this approach is the cost of scaling up operations sufficiently to yield the large volumes of hemoglobin required for routine transfusions.

Several other problems must be addressed before HBOCs can become broadly useful transfusion products. Erythrocytes exert little if any colloidal osmotic pressure, whereas hemoglobin (like other plasma proteins) has a colloidal osmotic effect. Cell-free hemoglobin thus acts as a plasma expander, because the colloidal osmotic pressure it exerts alters the intravascular volume by more than the volume transfused. Polyhemoglobin exerts much less colloidal osmotic pressure and can be prepared as an iso-oncotic solution (6). Some hemoglobin solutions also have vasopressor effects, that is, they increase blood pressure and decrease cardiac output. Several studies have indicated that this vasopressor effect is partly attributable to the ability of cell-free hemoglobin to scavenge nitric oxide, a cellular chemical messenger that stimulates blood vessel relaxation. Although this vasopressor effect is undesirable for most applications, it may turn out to be clinically advantageous for treating a small subpopulation of patients with septic shock who suffer from an uncontrollable decrease in blood pressure (7).

Perfluorocarbon-Based Products

For religious reasons, certain groups of people are unable to accept transfusions of either donor blood or human and animal proteins such as hemoglobin. For these patients, perfluorocarbons—molecules that are structurally similar to hydrocarbons except that hydrogen atoms are replaced with fluorine atoms—may be their only option if a blood transfusion is required. Perfluorocarbon liquids have an excellent capacity for carrying oxygen and carbon dioxide without actually binding to these gases (3). Through partial liquid ventilation of the lungs, perfluorocarbons have been successfully used to provide oxygen to premature infants with severe respiratory distress syndrome.

Perfluorocarbons provide an entirely different approach to oxygen transport. In cell-free hemoglobin solutions, oxygen is bound to hemoglobin in the same way as it is bound to the native molecule. In contrast, oxygen readily dissolves in the chemically inert perfluorocarbon liquid and can be easily extracted by oxygen-deprived tissues (Fig. 2). Perfluorocarbons are not miscible with aqueous solutions and must be prepared as emulsions before they can be used as blood substitutes. The oxygen loading capacity of perfluorocarbons is linearly related to the partial pressure of oxygen in equilibrium with the emulsion. Thus, at a given partial pressure of oxygen, hemoglobin binds significantly more oxygen than can be dissolved in the perfluorocarbon (Fig. 2). One perfluorocarbon, Fluosol DA,
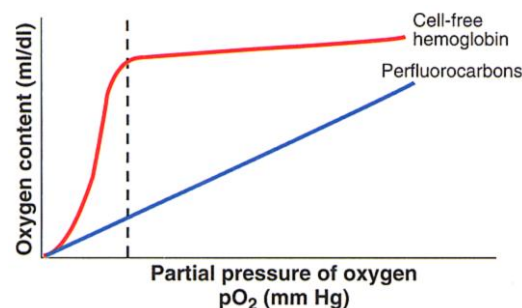
Fig. 2. Oxygen dissociation curves for cell-free hemoglobin and for a perfluorocarbon emulsion. Oxygen is bound to cell-free hemoglobin in the same way as it is bound to native hemoglobin. In contrast, oxygen readily dissolves in, but does not bind to, perfluorocarbon liquids. The oxygen loading capacity of perfluorocarbons is linearly related to the partial pressure of oxygen in equilibrium with the perfluorocarbon. At a given partial pressure of oxygen, hemoglobin binds more oxygen than can be dissolved in the perfluorocarbon.

has been approved by the FDA as a blood substitute for heart surgery: The fully oxygenated perfluorocarbon is infused at the time of coronary angioplasty to provide oxygen delivery during this surgical procedure. Even though the perfluorocarbon reduced cardiac trauma and pain during surgery (5), its use has never been fully embraced by physicians. Currently, Oxygent, a perfluorocarbon blood substitute developed by Alliance Pharmaceuticals, is in stage II/III clinical trials in the United States.

Risk Versus Benefit

New testing and screening procedures have rendered the donor blood supply increasingly safe. For example, the risk of transfusion-associated HIV infection is now estimated to be as low as 1 per 835,000 transfused patients

(8). Similarly, the risk of transfusion-associated infection with hepatitis C virus (HCV) is between 1 per 300,000 and 1 per 600,000, compared with an incidence of 1 per 103,000 in the early 1990s before a test for HCV became available. As the safety of the donor blood supply continues to improve, there must be careful consideration of the advantages of blood substitutes over donated blood, particularly given that new blood substitutes potentially carry unknown risks. Yet a shortage of donor blood for transfusion still advocates for the development of readily available blood substitutes. However, there is still a long way to go before artificial blood can replace real blood in routine transfusions. Most important, the intravascular dwell times of blood substitutes need to be increased, the cost of these products needs to be competi-

tive, and difficulties with obtaining and processing sufficient amounts of these compounds must be overcome.

References
1. J. McCullough, Transfusion Medicine (McGraw-Hill, New York, 1998), pp. 91–93.
2. E. C. Rossi et al., in Principles of Transfusion Medicine, E. C. Rossi et al., Eds. (Williams & Wilkins, Baltimore, 1991), pp. 1–11.
3. S. E. Hill, Can. J. Anaesth. 48, 532 (2001).
4. S. F. Rabiner et al., Ann. Surg. 171, 615 (1970).
5. R. M. Winslow, in Scientific Basis of Transfusion Medicine, K. C. Anderson, P. M. Ness, Eds. (Saunders, Philadelphia, 2000), pp. 588–598.
6. T. M. S. Chang, Blood Substitutes: Principles, Methods, Products and Clinical Trials, Vol. 1 (Karger, Basel, 1997) (www.medicine.mcgill.ca/artcell/bldsubcr.htm).
7. J. Creteur et al., Crit. Care Med. 28, 3025 (2000).
8. E. Vanvakas, in Evidence-Based Practice of Transfusion Medicine (AABB Press, Bethesda, MD, 2001), pp. 101–120.

VIEWPOINT

A Bioartificial Liver—State of the Art

Alastair J. Strain* and James M. Neuberger

End-stage liver disease is treated by liver transplantation, but donor organ shortages remain a serious problem. This has prompted the design of bioartificial liver devices to "bridge" patients until they either recover or receive a liver transplant. In these devices, patient plasma is circulated extracorporeally through a bioreactor that houses liver cells (hepatocytes) sandwiched between artificial plates or capillaries.

The healthy liver is able to regenerate itself after acute injury, but once damaged by fibrosis and cirrhosis—caused by a variety of chronic conditions such as alcohol abuse or infection with hepatitis virus B or C—it can no longer regenerate normally (1). Liver transplantation is a routine treatment for end-stage liver disease, but donor organ shortages remain a serious problem. Many patients with acute liver failure die while waiting for a transplant, and those with chronic disease often deteriorate so much that their survival rate after transplantation is low. Remarkably, "supporting" acute liver failure patients until their own liver repairs itself may negate the need for a liver transplant (2, 3). This has generated interest in designing a "bridging" device that would support or replace normal liver function until the patient's own liver recovered or a donor liver became available for transplant. Alternatively, a bridging device could support a failing liver long-term, in the same way that

Schools of Biosciences and Medicine, University of Birmingham, and Liver and Hepatobiliary Unit, University Hospital Birmingham, Edgbaston, Birmingham B15 2TH, UK.

*To whom correspondence should be addressed. E-mail: a.j.strain@bham.ac.uk

dialysis supports the failing kidney (2, 3). The principal goal is to develop a bioartificial liver (BAL) device in which patient plasma is circulated extracorporeally through a bioreactor that houses metabolically active liver cells (hepatocytes) sandwiched between artificial plates or capillaries.

What must a BAL bioreactor accomplish? The liver has a number of crucial functions that are principally carried out by hepatocytes. These cells synthesize many proteins, including clotting factors; they produce bile and regulate carbohydrate, fat, and protein metabolism; they detoxify the ammonia product of nitrogen metabolism and break down alcohol and drugs. In addition, the liver has Kupffer cells that are part of the immune system. The problem is deciding which liver functions are the most important and should be carried out by the BAL bioreactor. Biosynthetic capacity is perhaps the least essential because most proteins synthesized by the liver can be given exogenously to patients. The generation and detoxification of ammonia is undoubtedly important. Although an increase in ammonia in the blood is toxic to the central nervous system, reduction of ammonia levels per se is not sufficient to alleviate this toxicity (4). In response to this panoply of different functions, researchers

are attempting to develop BAL devices in which hepatocytes are optimally maintained so that they carry out as many activities as possible.

The Best Type of Cell for a BAL Bioreactor

Constituting 70% of the cellular content of the liver, the primary hepatocyte is clearly the cell of choice for a BAL bioreactor, particularly as it carries out most of the liver's functions. However, when removed from the complex architecture of the liver, hepatocytes rapidly lose liver-specific gene expression and become phenotypically unstable in culture. By manipulating tissue culture conditions—providing an extracellular matrix and growth factors, and coculturing hepatocytes with other types of liver cells—much can be done to maintain hepatocyte stability in vitro (5). Indeed, cross-talk among hepatocytes (which form the liver parenchyma), nonparenchymal liver cells, and bile duct epithelial cells is important for optimal hepatic activity (5) (Fig. 1).

The enormous scale-up required to use BAL devices clinically is problematic: At least 10^{10} hepatocytes in a BAL bioreactor would be needed to support a patient's failing liver. Given that the proliferative capacity of primary human hepatocytes and the ability to cryopreserve them is limited, there is a serious numbers problem (5). In short, there is insufficient good-quality human liver tissue from which to derive hepatocytes for routine clinical use in a BAL device. One alternative is to use hepatocytes from other species, par-