Alternate Sources and Substitutes for Therapeutic Blood Components

By Richard A. Kahn, Robert W. Allen, and Joseph Baldassare

The use of human blood components has three major drawbacks: their availability is often limited, some blood products are vectors for infectious diseases, and the procurement and processing of whole blood required to obtain a transfusable product is time-consuming and costly. As an alternative to human-derived blood components, substitutes and alternate sources of plasma proteins are being investigated. Although it will be many years before any product will be approved for human use, substantial progress has been made over the last few years.

RBC SUBSTITUTES

Several approaches have been taken to develop a clinically suitable RBC substitute.

Perfluorochemicals

Perfluorochemicals (PFCs) are large organic compounds in which all of the hydrogen atoms have been replaced by fluorine atoms. They are chemically inert and are not metabolized. Unlike other RBC substitutes, oxygen transported by a PFC is carried in solution. A PFC solution can dissolve 40% to 70% oxygen per unit volume, almost three times the oxygen-carrying capacity of blood.

The first report that a PFC could transport meaningful quantities of oxygen was published by Clark and Gollan, who showed that mice submerged in an oxygenated liquid PFC could survive. Subsequently, they showed that rat hearts could be maintained by perfusing with an oxygenated PFC followed by an aqueous perfusion fluid. Sloviter and Kamimoto dispersed PFC in serum and sustained a perfused rat brain for several hours.

Pure PFCs are immiscible with blood. Sloviter and co-workers, however, demonstrated that PFCs could be emulsified with surfactants and then mixed with blood without difficulty. Geyer later reported that rats kept in a high-oxygen atmosphere could be totally perfused with the PFC perfluorotributylamine, also called FC-47. Following perfusion, these “bloodless” rats survived as long as eight hours without being given plasma proteins or blood cells. Further studies by Geyer showed that by changing the composition of the blood substitute, altering its flow characteristics, and introducing a volume expander, rats survived the removal of all blood constituents and appeared normal.

Because the PFC must be used in an emulsified form, the formulation and preparation of emulsions are central to studies on these blood substitutes. The emulsifying agent most widely used in dispersing PFCs is the non-ionic detergent Pluronic F68 (BASF Wyandotte Corp, Wyandotte, Mich). Following emulsification, the preparation has the appearance of either a clear solution or a suspension resembling diluted milk, with particle sizes of 0.1 to 0.2 microns. The emulsification and particle size have important repercussions on the stability and potential toxicity of PFCs. Larger particles are more quickly excreted, but are difficult to prepare in stable emulsions, and have been shown to aggregate in the liver and spleen. Thus, PFCs that are rapidly eliminated from the circulation do not form stable emulsions, while PFCs that are easily emulsified have long body retention times.

In an effort to find both stable emulsion and rapid clearance in a single PFC, there has been a systematic effort to screen a great number of PFCs. The most successful preparation, Fluosol-DA (Green Cross Corp, Osaka, Japan), consists of 20% (wt/vol) PFCs: perfluorodecalin (14 g/dL), with a half-life of 7.5 days, but with only a moderate emulsification capacity, and perfluorotributylamine (6 g/dL), with a half-life of 65 days, but excellent emulsification. In addition to the two PFCs being emulsified with Pluronic F-68 in a balanced salt solution, hydroxyethyl starch is added to maintain blood volume and to enhance the flow characteristics of the solution. The preparation has a circulation half-life of about 13 hours and a tissue half-life of nine days. This new PFC combination was distributed by...
the Japanese Green Cross (Osaka, Japan) to its American subsidiary Alpha Therapeutics (Glendale, Calif) and has been used in the United States in the most recent widely publicized patient studies.

In 1979, the first adult volunteers were given the PFC emulsion Fluosol-DA. The initial clinical studies were performed in Japan and indicated a high degree of safety and efficacy.\(^9\)\(^{11}\) Mitsuno et al\(^{14}\) administered Fluosol-DA (6 to 25 mL/kg) to 186 Japanese patients and did not observe any untoward reactions; measurements of oxygen delivery and consumption indicated a beneficial effect of the infusion.

In April 1980, clinical trials using Fluosol-DA were initiated in the United States. The first clinical report of the effects of PFC administration to patients in this country was provided by Tremper et al.\(^{15}\) Seven severely anemic patients were given Fluosol-DA before surgery. When low levels of supplemental oxygen were used, the PFC had virtually no benefit. When patients received pure oxygen, their arterial oxygen content rose significantly and the PFC provided 24\% of the oxygen consumed.

**Advantages and disadvantaged of PFCs.** Because they are biologically inert, PFCs can be given in the same concentration as conventionally used electrolyte solutions. They contain no antigens, and therefore, typing or cross-matching between donor and recipient is unnecessary. They are also easily synthesized from readily available materials and are free of infectious diseases.

PFCs also have advantages other than as substitutes for blood. For example, since they do not carry carbon monoxide, PFC administration could provide oxygen to a carbon monoxide victim until the patient replaced abnormal red cells. Another potential use relates to the PFCs' small particle size, which enables the suspension to penetrate occluded vessels, as in cerebral ischemia or myocardial infarction.

Because PFCs do not preferentially extract oxygen from the air as does hemoglobin, the oxygen level in a PFC solution equilibrates with the oxygen level in the atmosphere. PFC therapy therefore requires the concurrent administration of 60\% to 100\% oxygen.\(^{11}\) A high-oxygen environment can render the patient vulnerable to oxygen toxicity, especially when the concentration of oxygen in inspired gas is above 70\% for more than 24 hours.\(^{14}\) Thus, the advantages of PFCs (ie, increased oxygen-carrying capacity) must be evaluated against the potential oxygen toxicity. This disadvantage may prove to be the limiting factor in PFCs use as a substitute for blood transfusion. Of note, the observation by Gould et al,\(^{15}\) that plasma alone is an effective oxygen carrier if animals inspire 100\% oxygen, raises the question of whether the benefits seen with PFC infusion are really due to the substitute itself.

Another disadvantage of PFC emulsions is their instability, resulting in the need to store them frozen. Clark et al,\(^{16}\) however, recently studied a surfactant that allows stable emulsions at room temperature with a variety of PFCs. An improvement in storage properties would be a major achievement; PFC solutions could be handled like standard intravenous solutions. Additionally, this emulsifier enables the PFC concentration to be increased to over 50\% (compared to current PFC concentrations of 20\%), enabling less PFC and less oxygen to be given while achieving the same benefit obtained with current preparations.

A third disadvantage relates to PFC retention by the liver and spleen.\(^{17}\) One patient, who received 4 L of PFC over a 12-day period and died four months later, was found at autopsy to have substantial morphological evidence of PFC retention.\(^{18}\) Because the reticuloendothelial system (RES) is involved in PFC clearance, saturation of the RES may reduce the body’s ability to clear other foreign substances, notably bacteria or viruses. In fact, Lutz and co-workers\(^{19}\) showed that in mice challenged with bacterial toxin given simultaneously with PFC administration, the lethality of the toxin rose nearly eightfold.

Finally, early studies on PFC infusions noted adverse pulmonary reactions that were attributed to physical properties of the PFC\(^{15,20}\) which affected either platelets or plasma proteins.\(^{21}\) For example, it has been suggested that Fluosol-DA infusion activates the complement system, resulting in the aggregation of pulmonary macrophages\(^{12,22}\) and consequent pulmonary congestion and hypoxemia. More recently, Bucaja et al\(^{23}\) studied the effect of another commercially available PFC emulsion on macrophages and found the material to be cytotoxic; 24-hour exposure to a 15\% concentration of PFC nearly eliminated macrophage integrity. Virmani et al\(^{24}\) showed that PFC emulsions disrupted the phagocytic function of neutrophils and monocytes. These reports clearly suggest that the mechanism and consequences of leukocyte dysfunction following exposure to PFC preparation require more detailed evaluation.

The uncertainties surrounding the use of PFCs prompted the Blood Products Advisory Committee of the Food and Drug Administration to disapprove Fluosol-DA for human use.\(^{25}\) The potential toxicity of the high O\(_2\) environment in which PFC is administered, the relative paucity of data regarding the effect of Fluosol’s emulsant (Pluronic F68) on the recipient’s blood cells and tissues, and, most important, the lack of convincing data that the PFC itself was efficacious were key factors contributing to the disapproval.

In summary, current PFC emulsions are unsatisfactory for use as blood substitutes; their safety is not established and their utility is hampered by the requirement of a high-oxygen environment. Even if the safety concerns were eventually resolved, the use of PFCs would likely be limited to patients with religious objections to blood transfusions or to emergency treatment in a controlled hospital setting.

**Hemoglobin Solutions**

Early studies on the use of unpurified hemoglobin solutions for transfusion were often associated with renal abnormalities and coagulation defects which precluded their use.\(^{26-28}\) In 1967, a major improvement was made by Rabiner and colleagues,\(^{29}\) who suggested that removal of RBC membrane fragments (ie, stroma) from hemolyzed RBCs would eliminate the renal and coagulation problems. Later, Birndorf and co-workers\(^{30}\) and Cochin et al\(^{31}\) confirmed the undesirable effects of stroma in hemoglobin solutions.

The methodology used by Rabiner et al\(^{30,33,34}\) consisted of
slowly lysing washed RBCs with a buffered solution of water, followed by high-speed centriugation and micropore filtration. This technique fragmented the RBC membrane into relatively large pieces which could be removed easily; “free” hemoglobin prepared by this methodology was well tolerated in dogs34,36 and monkeys.31

Moss et al.,37 however, using a similar preparation, reported alterations in in vitro clotting tests. Savitsky et al38 infused hemoglobin prepared by the Rabiner method into volunteers and noted transient alterations in cardiovascular, renal, and blood coagulation parameters; however, by 24 hours after infusion the abnormalities were corrected. They also showed that the Rabiner preparation was not “stroma-free,” but that it contained a small amount of lipids likely derived from the RBC membrane. In attempts to purify hemoglobin solutions further, the conditions of hemolysis, centrifugation, and filtration have been modified39,40; hemoglobin has been crystallized free of both soluble and insoluble contaminants,41 or the crude solution acidified to precipitate the stroma.42 Sehgal et al43 have attempted to increase production of stroma-free hemoglobin using high-pressure lysis of RBCs and acid precipitation, followed by centrifugation and micropore filtration to remove stroma. Although they could prepare 20-L batches of solution, the preparation was still contaminated with lipids. Other procedures for increased production have been developed,44 but analyses of the final solutions have not been published.

Adverse effects. Although highly purified hemoglobin solutions can be prepared, questions regarding safety still remain. Bolin and co-workers45 infused purified hemoglobin into dogs, monkeys, and pigs and saw thrombocytopenia and disseminated intravascular coagulation (DIC) develop in the first few hours after infusion in dogs, but not in monkeys and pigs. Savitsky et al38 reported variations in the response to infusion of a hemoglobin solution into eight normal volunteers, and other investigators have noted unexplainable variations in the toxicity seen between preparations made using the same technique (R. Bolin, Letterman Army Institute of Research, San Francisco, personal communication, March 1984). These results raise the question of contaminants below detectable levels which cause adverse reactions. In addition, the differences in response among animal species make interpretation of safety data confusing. Finally, it is possible that hemoglobin itself is toxic under certain conditions. Data supporting this hypothesis have been obtained by Wilson and Thomas,46 who demonstrated that hemoglobin can activate the complement system, although it is possible that the preparation used was impure.

Obtaining a nontoxic hemoglobin solution is one difficulty that has prevented the clinical use of these preparations. Other problems include the short half-life of free hemoglobin in the circulation,30,34,47 its significant oncotic effect, and its unacceptable high affinity for oxygen.48,49

Free hemoglobin’s oncotic effect limits the maximum amount that can be given; only 7 g/dL of free hemoglobin can be administered as a 1:1 replacement for blood without exceeding the normal oncotic pressure of plasma. Increasing the amount infused raises the risk of volume overload in the recipient. Significantly less oxygen transport capacity is attainable with hemoglobin in solution when compared to an equal volume of blood, and thus the hemoglobin solution cannot be used as a 1:1 replacement fluid.

In order to stabilize the hemoglobin molecule, as well as to decrease its oncotic effect, several approaches have been taken. One is to crosslink (or “modify”) hemoglobin molecules48 either intermolecularly or intramolecularly. Although the intravascular half-life of crosslinked hemoglobin is prolonged (to 15 to 30 hours) and the oncotic effect of the polymer is similar to hemoglobin in RBCs, the oxygen affinity of modified hemoglobin remains extremely high. Additionally, many modifying agents (eg, glutaraldehyde) crosslink hemoglobin molecules both intra- and intermolecularly, resulting in an assortment of hemoglobin polymers. Batch-to-batch reproducibility is difficult, and the potential adverse effects of various polymeric forms are difficult to assess. One promising new crosslinking agent is 3,5-dibromo-salicyl-bis-fumurate,50 a nontoxic inexpensive reagent capable of reproducibly crosslinking hemoglobin at specific sites. Another approach is to stabilize hemoglobin by attaching it to a larger molecule that persists in the circulation for longer periods. Tarn et al51 and Cerny et al52 attached hemoglobin to dextran and noted longer circulation times, but the high viscosity of this complex limits the amount that can be given.

The most successful attempts to normalize the $P_50$ of hemoglobin solutions have used pyridoxal 5'-phosphate (P5-P), an organic phosphate analogue of 2,3-diphosphoglycerate53 which decreases the oxygen affinity of hemoglobin to that of RBCs (ie, 26 to 28 torr). To achieve a $P_50$ approximating that of RBCs and a longer circulation time, many investigators have both pyridoxalated and crosslinked hemoglobin. DeVenuto and co-workers54 pyridoxalated and polymerized hemoglobin with gluteraldehyde, and achieved a half-life of 25 hours and a $P_50$ of 19 to 22 torr in the rat model. Similar results were achieved in baboons by Sehgal et al.55 Other investigators tested pyridoxalated fumarate-stabilized hemoglobin and achieved a $P_50$ of 28 to 30 torr with a half-life of 20 hours in rabbits.56 Pyridoxalated polymerized hemoglobin does not have the oncotic pressure restrictions of unmodified hemoglobin and can be administered in a concentration of 14 to 15 g/dL, the hemoglobin concentration in whole blood.

The $P_50$ of these preparations is a composite $P_50$ because not all of the hemoglobin will be pyridoxylated and crosslinked in a given preparation. Because polymerized hemoglobin and unmodified hemoglobin are eliminated by different mechanisms,56 the contribution of each to any toxicity seen will be difficult to resolve. For this reason, future studies are likely to focus on methods for producing homogeneous preparations of modified hemoglobin which could be used to determine the etiology of toxic effects.

How well do the pyridoxalated polymerized preparations replace blood as an oxygen transport solution? DeVenuto and Zegna used almost totally exchanged rats and Moores et al57 used a 50% exchange in pigs and dogs. It was concluded that the modified-hemoglobin transfusion was essential with the rats, and of substantial clinical benefit with the pigs and dogs. The Moores study noted that in a partial
blood exchange (a situation more likely to simulate clinical conditions), the importance of the hemoglobin solution in oxygen delivery is far less than in the highly unnatural totally exchanged animal models. Moores also noted that modified hemoglobin was no better than unmodified preparations despite the former’s better $P_{50}$ and longer half-life in the circulation, implying that the presence of hemoglobin may be more important than its $P_{50}$.

Moreover, it has been shown that animals exchanged to hematocrits above 10% can survive with a colloid as the infusate. At hematocrits above 15%, the contribution of the transfused hemoglobin to total oxygen consumption may be nil. This does not prove that hemoglobin solutions are of little value, but rather that the clinical efficacy of one preparation over another will be difficult to evaluate. If the toxicity issues can be resolved, current formulations (ie, pyridoxalated polymerized or tetrameric stabilized) may be of clinical use. Because they are excellent volume expanders and can contribute directly to oxygen delivery, they are a good choice for an emergency resuscitative fluid.

Other problems. Other potential problems with hemoglobin solutions relate to the source of the hemoglobin, its storage, and the production of an immune response.

Hemoglobin solutions are currently being made from outdated human blood. If all of the outdated blood in the country were channeled into hemoglobin solution production, approximately 600,000 units would be available (5% of the 12 million units collected). Because it is unlikely that all units of outdated blood could be salvaged, it is uncertain whether this source would satisfy acute use and provide emergency reserves.

An alternate source for hemoglobin is animal blood. DeVenuto and Zegna have shown that crosslinked and pyridoxalated bovine hemoglobin has the equivalent circulatory time and oncotic pressure of its human counterpart. Moreover, it has a more favorable $P_{50}$ than human hemoglobin. On the other hand, it is unknown if there is an immunogenic response of humans to bovine hemoglobin, or even if purified human hemoglobin itself is immunogenic.

A final concern is the stability of hemoglobin during storage. Several studies have shown that there is no loss of activity at 4 °C for at least five months and maybe up to a year. While refrigerated or frozen storage may be suitable for hospital use, it is unsatisfactory for use as an emergency resuscitative fluid, particularly in the battlefield, and a freeze-dried powder would be more suitable. Greenberg et al have shown that lyophilized hemoglobin could be kept for 2.5 months at room temperature, six months at 4 °C, and one year if frozen. Other investigators, however, have concluded that purified hemoglobin is unstable in the dried state, and the presence of stabilizers was necessary to prevent deterioration. None of these studies used the pyridoxylated polymerized preparations, and in vivo studies were limited. Because the freeze-drying procedures were empirically derived and small batches of hemoglobin were used, it is uncertain whether successful long-term storage of clinically suitable freeze-dried hemoglobin is feasible.

Clinical studies. There have been 14 published reports in which a substantial volume of hemoglobin solution was transfused into humans. All but Savitsky et al used a crude uncharacterized preparation of hemoglobin administered to normal volunteers, and in all of the reports, adverse effects were noted. In the Savitsky study, a micro-pore-filtered hemoglobin solution was given to eight normal volunteers, all of whom experienced cardiovascular, renal, and coagulation abnormalities which lasted several hours. Since 1978, no clinical trial has been initiated in the United States.

To summarize, the major shortcoming of hemoglobin solutions as blood substitutes is their short half-life. With a half-life of only one day, the usual recipient of a blood transfusion will require repeated transfusions. Thus, hemoglobin solutions may be limited to use as an emergency resuscitative fluid.

The benefit of using a hemoglobin solution must be weighed against its “cost” both in dollars and safety. The sporadic adverse reactions seen in animals remain an enigma; studies are needed to clarify the effect of hemoglobin solutions on the kidney, liver, RES, immune, and blood coagulation systems.

Encapsulated Hemoglobin

Encapsulation of hemoglobin in liposomes is an alternative approach to preparing an RBC substitute. Liposomes can be prepared from a single phospholipid, a mixture of phospholipids, or mixtures of phospholipids and neutral lipids, eg, phosphatidylcholine plus cholesterol. The properties of liposomes can be somewhat controlled by using different lipids and/or different methods of preparation. For example, liposomes can be multilamellar (ie, composed of a series of alternating lipid layers separated from each other by an aqueous compartment) or unilamellar and large or small.

Although the majority of the research on liposomes has focused on their roles in cell membrane function or as carriers of drugs, there has been some work using liposomes as a blood substitute. Hemoglobin has been successfully encapsulated in lipid vesicles at concentrations equal to that found in RBCs. Most workers have encapsulated concentrated hemoglobin solutions in liposomes made by mixing phospholipids and cholesterol. The liposomes contained no protein, either inside or within the lipid bilayer, other than the encapsulated hemoglobin and did not cause an immune reaction. The functional characteristics of liposomal substitutes were similar to those of native red cells; their oxygen dissociation curve was similar to whole blood, with $P_{50}$ values of 26 to 28 torr.

A different approach to the preparation of vesicles was taken by Davis and Asher, who encapsulated stroma-free hemoglobin in a polymerized hemoglobin “membrane.” The crosslinked membrane was permeable to oxygen and impermeable to hemoglobin in solution. These vesicles were capable of reversibly binding oxygen and were less than 4 μm in diameter. Moreover, the vesicles were reported to be stable under conditions of normal blood flow. Liposomes thus appear to be at least equal to other artificial blood substitutes in their ability to transport and deliver oxygen.
Many investigators\textsuperscript{73-75} have used rats to perform exchange transfusions with liposomal suspensions. Djordjevich and Miller\textsuperscript{73} found that complete replacement of RBCs with liposomal suspensions sustained life for several hours with no adverse reactions. Hunt and Burnett\textsuperscript{74} found that their preparation could sustain rats for up to seven hours in an exchange transmission. Ivankovich and Djordjevich\textsuperscript{75} reported that rats survived 90\% exchange transfusion for an average of 14.7 hours and up to 28.5 hours after liposomes were given, breathing only room air. It should be remembered, however, that a $<95\%$ exchange transfusion in animals can be tolerated simply by administering a colloid or crystalloid solution.

Although these studies are encouraging, many problems remain. For example, the rapid rate of liposome removal from the circulation must be modified (the half-life is only five hours).\textsuperscript{76} Studies of the distribution of liposomes after injection indicate that the liver is the site of liposome retention,\textsuperscript{77-81} containing approximately 50\% of the injected dose within a few hours after administration. Rapid uptake of liposomes not only results in shortened liposome lifetime, but could also result in reticuloendothelial blockage. Profitt et al\textsuperscript{78} pretreated mice with liposomes to block the RES and showed that the distribution of liposomes added after RES blockage could be altered. Thus, saturation of the RES could be a serious problem for patients with a compromised immune system.

Recently, natural and synthetic glycolipids have been inserted into the liposomal membrane and tested for their ability to alter liposome clearance. Incorporation of these glycolipids decreased uptake by the RES.\textsuperscript{82-84} Many natural glycolipids are antigenic, however, and thus would not be suitable for insertion into a liposome to be transfused. Hunt and Burnett\textsuperscript{84} attached inert carbohydrates to lipids and claim to reduce RES clearance and binding of liposomes to tissues. Thus, addition of inert carbohydrates may be one way to retard rapid liposome clearance.

The potential toxicity of a liposomal preparation is also of concern. Initial studies\textsuperscript{73-76} indicated that hemoglobin-filled liposomes were nontoxic when infused into rats or rabbits. These studies, however, did not address the long-term effects of the liposomal preparations. Also, toxicity of liposomes could result from impurities in the hemoglobin preparation, the lipids themselves, or impurities introduced in the encapsulation procedure, which could be toxic if the contents were to escape.

A possible solution to many of the problems discussed may lie in the use of synthetically made vesicles, or so-called “membrane mimetic systems.”\textsuperscript{85,86} Such vesicles can be stabilized by polymerization, and have a shelf life of several months. Moreover, the size of the vesicles and their permeability properties can be engineered as desired. At this time, however, the biological applications of these vesicles have only begun to be determined.

In summary, the use of encapsulated hemoglobin as an RBC substitute has received relatively little attention, particularly in comparison to the work on PFCs and hemoglobin solutions. While the technology is still in its infancy, encapsulation appears to be the approach most likely to lead to a blood substitute that has all the properties of RBCs. Major obstacles remain, however, particularly the toxicity, stability, and storage of such preparations. In addition, there is uncertainty as to whether mass production can be cost-effective.

**ARTIFICIAL PLATELETS**

The complex role of platelets in hemostasis is poorly understood, and it is not currently possible to fashion an artificial platelet that will mimic platelet function. Nevertheless, several approaches have been explored as means to develop a suitable platelet substitute.

A number of investigators have reconstituted one or more of the major platelet membrane glycoproteins into lipid vesicles.\textsuperscript{87-89} Although some platelet function was attained, the preparations could not in any fashion be considered platelet substitutes. Virtually all of the problems that were discussed in relation to liposomes as artificial red cells are also relevant for the development of liposomes as platelet substitutes. In addition, few of the membrane proteins critical to platelet function have been identified or isolated, and it would be critical to orient these proteins properly in the lipid bilayer.

Another approach has been to examine the efficacy of freeze-dried or fragmented platelets. Although such preparations have been used clinically,\textsuperscript{90,91} the results have been challenged by animal studies; three independent reports showed that both freeze-dried or fragmented platelets were of no value in the control of bleeding.\textsuperscript{92,94}

When the role of platelet phospholipids in the control of bleeding was uncertain, Schulman and associates\textsuperscript{95} administered soybean phospholipids to leukemic children who were thrombocytopenic. Although they found a reduction in bleeding in four of seven patients, a subsequent study in animals showed that phospholipids were ineffective,\textsuperscript{94} and there have been no further reports on the use of this material.

Although early investigations on the search for a platelet substitute proved unfruitful, recent work with liposomes provides some encouragement. An enormous number of problems remain, however, not the least of which is the incomplete understanding of the biochemistry of platelet function. The complexity of the platelets will undoubtedly make construction of a complete substitute extremely difficult.

**IN VITRO STEM CELL CULTURING**

It is conceivable that pluripotent stem cells could be maintained in culture to replicate and differentiate into mature blood cells. Provision of the necessary growth factors and an efficient method for harvesting functional cells would be necessary to make this a cost-effective approach.

Based on the culture system of Dexter et al,\textsuperscript{96} a number of investigators have successfully established long-term cultures of human bone marrow cells.\textsuperscript{97-99} The cultures survived less than six months, however, and it is uncertain whether there was significant stem cell replication. In addition, while
differentiation was seen, it did not proceed along all cell lines; granulocytes were produced, but mature megakaryocytes were rarely seen. Also, the list of culture supplements is complex and variable and there is little knowledge of critical components or their mechanism of action. Finally, cultures of mouse stem cells live far longer than their human counterparts and it is uncertain whether the differences represent suboptimal conditions or are unique to the species. A greater understanding of the events governing hematopoiesis is essential, including the processes that regulate the balance between self-renewal and differentiation. Other major problems that remain include production of sufficient numbers of cells (10⁹ to 10¹⁰) for transfection, and the harvest of mature functional cells free of contaminants. Finally, compatibility testing between “donor” and recipient will probably be necessary, thus negating the advantage of a universally compatible product.

In summary, stem cell cultures offer the potential of producing transfusable blood cells in a laboratory environment. Whether the procedure can be expanded to produce sufficient numbers of functional cells in a cost-effective manner is uncertain. Because much basic information regarding the control of stem cell replication and differentiation is lacking, more research is needed before an accurate assessment of stem cell culture usefulness can be made.

ALTERNATE SOURCE FOR PLASMA PRODUCTS

Plasma proteins are currently separated from plasma by routine biochemical fractionation techniques. They have the potential, however, of being produced by micro-organisms carrying the genetic code for these proteins.

Application of Recombinant DNA Technology to the Large-Scale Production of Plasma Proteins

The major proteins currently recovered from plasma fractionation are albumin, factor VIII, prothrombin complex, and immunoglobulins. Although the plasma fractionation industry supplies the need for these products in the United States, the world need has not been met. Furthermore, other proteins cannot be recovered in high yield from plasma, or can be recovered only at the expense of another product. For these reasons and those mentioned at the beginning of this paper, recombinant DNA technology is believed to hold much promise as an alternate source of plasma proteins.

A major challenge facing gene-cloning technology is the expansion of production to meet the demand. For example, about 6 million L of plasma was fractionated in the United States in 1982, 3.5 million L was fractionated to meet US demands for plasma products. Therefore, large-scale production and purification capabilities must be available before genetic engineering can meet even the US demand for plasma products. For some plasma products (eg, albumin), thousands of kilograms of protein will have to be synthesized and purified. The demand for some products (eg, factor VIII) will not require such large amounts to be synthesized, but purification schemes that will not inactivate the proteins will have to be developed. An additional problem is the design of biological systems for the post-translational modification of those plasma proteins that require such modifications for activity. If the gene that codes for a glycosylated plasma protein is cloned in a bacterial cell, which is incapable of protein glycosylation, a means of glycosylating the purified polypeptide may need to be devised. Alternatively, the gene may be cloned into yeast or mammalian cells, which may correctly glycosylate the polypeptide. Other post-translational modifications may also be necessary for protein activity.

Each plasma protein will present its own collection of specific problems to the recombinant DNA industry. In the following discussion, the plasma proteins of demonstrated or potential clinical value are identified and the general status of cloning the gene that codes for each protein is reviewed.

Albumin. The gene for human albumin is one of the first genes to be cloned which codes for a plasma protein. Numerous recombinant DNA (rDNA) companies have claimed to have both recombinant yeast and bacterial strains carrying the human albumin gene. Genentech, Inc has published the detailed construction of a recombinant *Escherichia coli* that produces albumin which is biochemically and immunologically identical to native human albumin. However, the complex molecular configuration proposed for natural albumin by Brown and Dugaiczyk et al was not evaluated in the recombinant protein. Thus, the degree to which the natural and recombinant albumin molecules are truly identical, other than amino acid sequence and immunologic similarity, is unclear. Interestingly, the *E coli* appeared to synthesize a small amount of albumin which was smaller in size than the native product, and may have been incompletely synthesized albumin. Thus, not all of the protein synthesized by this particular strain is comparable to native protein. Other biotechnology companies claiming to have cloned the albumin gene have not published the biochemical properties of the product, its rate of synthesis by the host cell, or other technical information, all of which is regarded as proprietary.

When considering the feasibility of producing albumin by rDNA technology, the first obstacle to be faced is whether enough protein can be produced to meet present needs in a cost-effective manner. In 1982 the United States used approximately 87,500 kg of serum albumin, a rate of use of approximately 387 kg/10⁶ population/yr. To address the feasibility of producing sufficient albumin to meet US demand, as well as the additional 57,500 kg of albumin that was exported in 1982, it is important to know how much albumin could be synthesized by “state-of-the-art” recombinant strains of bacteria or yeast. Although no rDNA company will divulge synthetic rates for individual proteins due to the proprietary nature of such information, it is acknowledged that in current expression systems, the synthesis of a cloned gene product can constitute 10% to 50% of the total protein synthesized by the cell. The rate varies for individual proteins and the maximum achievable rate may vary significantly.

We estimate that 116.7 × 10⁶ L of fermented culture per year will be necessary to produce sufficient albumin to meet
the US demand, $193 \times 10^6$ L to supply the worldwide market. Calculations were based on the amount of protein that can be harvested from 1 L of fermented culture. Although it may seem difficult to produce such large amounts of fermented culture, the brewery industry in the United States easily produces such quantities.

Other investigators have arrived at a different conclusion; namely, that the current rDNA industry is not equipped to produce and process sufficient albumin to keep pace with worldwide, or even nationwide, demand. These authors assume that 50 gal of water will be needed to process each liter of ferment, because that much water is currently needed to process each liter of plasma by standard fractionation techniques. Based on the yield of recombinant albumin per gallon of water used, and extending water usage to an annual rate, they conclude that 10 billion gallons of water would be needed to produce the albumin currently fractionated from plasma with only 50 million gallons of water.

However, the assumptions of Drees et al regarding water usage are incorrect, because the entire contents of the fermentor will not have to be processed. Processing recombinant E coli is not the same as plasma fractionation, because only the bacteria need to be processed and not the media and bacteria both. Separation of bacteria from media can be easily achieved by continuous flow centrifugation, which requires very little water.

The cost of the final product will be dependent on its rate of synthesis and how the product is recovered from the recombinant organism. Perhaps the greatest difficulty in producing clinically acceptable recombinant albumin will be purification of protein uncontaminated by bacteria. One possible method to increase the efficiency of production involves the use of non-pathogenic strains of bacteria such as Bacillus subtilis or yeast. B subtilis has been used industrially for the large-scale production of other proteins and is attractive because of its great biosynthetic capability (ten to 15 times the amount of protein synthesized by an E coli culture), and the fact that the products made by the cell are excreted into the medium rather than accumulated intracellularly as in E coli. These features would facilitate subsequent purification and processing, and allow continuous fermentation in chemostat culture. To date, however, the use of B subtilis for the production of recombinant proteins lags behind the E coli system.

Expression of recombinant albumin in yeast also lags behind the E coli system, yet offers other advantages. Yeast has been grown industrially for years and thus considerable experience exists in large-scale bioprocessing techniques. In addition, the wet weight yield of yeast in a fermentor is about 2½ times the wet weight yield of E coli. Since current strains of recombinant yeast can accumulate protein to a level comparable to E coli (P. Valenzuela, Chiron Corp, Emeryville, Calif, personal communication, February 1984), the yield of recombinant protein would be increased 2½ times per liter of ferment. Also, yeast has the capacity to secrete certain recombinant proteins into the medium.

In summary, current technology employing E coli is capable of producing recombinant albumin to meet worldwide demand. Attention is now focused on the purification and scaled-up production of recombinant albumin and whether this process can be cost-effective. Because E coli produces pyrogens and other antigenic molecules, the recombinant protein will require extensive purification. While standard plasma fractionation techniques may be applicable to the initial purification, more rigorous purification methods may be required before a clinically useful product is isolated. It is possible that more sophisticated purification techniques will be efficacious but not cost-effective. Alternatively, albumin may be produced in a more cost-effective manner in other cloning systems (eg, B subtilis) in which removal of hazardous contaminants may be less of a problem.

**Factor VIII.** Most factor VIII is currently obtained by fractionating fresh-frozen plasma. In the United States in 1982, approximately 500 million units of factor VIII were prepared by plasma fractionation. These 500 million units constitute only about 280 g of purified protein.

Information regarding the molecular nature of factor VIII has been slow to accumulate and, therefore, it is likely that the isolation of the mRNA coding for the molecule will be difficult. Consequently, the approach taken to obtain recombinant factor VIII has been to synthesize an oligonucleotide probe based on the amino acid sequence of the molecule, which is then used to screen bacteria containing human DNA. Using this technique, segments of human chromosomal DNA which code for portions of the molecule have been identified and these segments have been spliced together to construct the complete gene.

While a spliced factor VIII gene may be necessary for synthesis of the protein by bacteria or yeast cells, an intact chromosomal gene with its intron/exon organization may be suitable for expression by cloning systems that use mammalian cells. Techniques exist for the efficient introduction of foreign DNA into a variety of cultured mammalian cell lines, and results from several studies have shown that the genetic information encoded in a clone of human chromosomal DNA can be properly expressed by a mouse cell line. Because this approach may prove feasible, virtually all of the rDNA companies working on cloning factor VIII are studying mammalian expression systems.

Several problems can be anticipated in the synthesis of factor VIII by rDNA methods. First, the molecule is fairly large in size (200,000 mol wt), whereas the largest human protein efficiently made by bacteria has been albumin, with a molecular weight about one third that of factor VIII. It is possible that only a portion of the factor VIII:C molecule is required for the procoagulant activity of the entire molecule, so that DNA that codes for the smaller fragment alone could be cloned and efficiently expressed in a bacterial system.

Another potential problem is the fact that factor VIII is a glycoprotein, and it is still unclear whether the associated carbohydrate is important for procoagulant activity. If so, the factor VIII molecule synthesized by recombinant bacteria may somehow have to be glycosylated before it is clinically effective. Alternatively, the gene could be cloned and expressed using hosts capable of glycosylating proteins (eg, yeast or mammalian cells).
A final problem is the inherent instability of the molecule. Factor VIII is extremely sensitive to proteases and thus it will be essential to insure that organisms synthesizing the molecule have low endogenous levels of proteases. Furthermore, because disrupting virtually any cell results in the release of proteases, it is desirable for a recombinant organism that is synthesizing factor VIII to secrete the molecule into the medium.

Several biotechnology companies have announced that recombinant factor VIII will be available soon, claiming that the functional portion of the molecule has been cloned and synthesized by a recombinant mammalian cell line. The lack of a complete understanding regarding the molecular structure and function of factor VIII:C, however, suggests that these estimates may be optimistic. Unless the key structural and functional features of the molecule are simpler than presently envisaged, recombinant factor VIII may not be commercially available for a number of years.

**Factor IX.** Several laboratories have reported cloning the gene that codes for factor IX.110–112 This gene was identified from a cDNA library constructed using human liver mRNA. An oligonucleotide probe was chemically synthesized based upon the known amino acid sequence of the protein. The oligonucleotide was subsequently radioactively labeled and used as a specific hybridization probe against the liver cDNA library. The cDNA clone isolated by Kurachi and Davie111 contained the entire coding sequence for factor IX. In addition to the identification of cDNA clones, Choo and colleagues116 have cloned the chromosomal DNA that codes for factor IX.

The development of recombinant-derived factor IX protein should be less difficult than that of factor VIII because the size of the polypeptide (57,000 mol wt) is within the limits of present recombinant technology. Furthermore, a report describing the cloning of the factor IX gene has been published111 and other clones have been constructed by rDNA companies. The production of recombinant factor IX will not be easy, however. The molecule is a glycoprotein and it is not known how the activity of factor IX is influenced by the associated carbohydrate. As with factor VIII, it may be necessary to clone the factor IX gene in yeast or mammalian cells capable of correctly glycosylating the protein. However, a large part of the carbohydrate associated with factor IX is attached to the 9,000-mol wt fragment, which is cleaved from the molecule as it is activated.113 Thus, the carbohydrate portion of factor IX may not function in coagulation. A more challenging obstacle to the production of functional recombinant factor IX involves the other major post-translational modifications of the protein that normally occur, eg, the conversion of several N-terminal glutamic acid molecules of factor IX to γ-hydroxylglutamate residues by a vitamin K-dependent enzyme system.114 This enzyme system may be lacking in bacterial and yeast hosts and therefore the glutamic acid residues would require chemical modification in order to produce a functional factor IX molecule.

In summary, there is not much interest in the production of recombinant factor IX because of the small market for the product and an abundant supply of factor IX concentrate from plasma fractionation. Thus, the availability of recombinant factor IX is likely to follow the development of recombinant factor VIII.

**Plasma enzyme inhibitors and plasminogen activator.** Plasma protease inhibitors have gained widespread attention for their potential as therapeutic agents. The plasma proteases of interest are α₁-antitrypsin, antithrombin III, and Cl-esterase inhibitor. As of 1982, only 2.5% of the plasma fractionated in the United States was devoted to the preparation of all inhibitor products.102

The gene that codes for α₁-antitrypsin has been cloned by at least one laboratory,115 which synthesized an oligonucleotide probe based upon the known amino acid sequence of the molecule and screened a cDNA library prepared from liver mRNA. Numerous clones were identified and several were found to synthesize α₁-antitrypsin. In addition, Chiron Corp has cloned the entire gene that codes for α₁-antitrypsin in yeast and *E. coli.116* In yeast, 20% of the total protein inside the cell during fermentation is α₁-antitrypsin. The potential market for α₁-antitrypsin, however, is uncertain.

Oligonucleotide probes that identify the antithrombin III gene have now been prepared in several laboratories. The cDNA that codes for the entire protein sequence and the entire chromosomal gene has been cloned in *E. coli.116–118* but not in other hosts. Problems associated with the production of recombinant antithrombin III should not present a unique challenge, other than the potential need for glycosylation of the protein.

Recent commercial interest in recombinant-derived plasminogen activator (PA) comes from the knowledge that infusion of the molecule can promote the dissolution of a thrombus.119 The PA used in this study was derived from a melanoma cell line that secretes PA into the medium.120 This PA was found to be identical to that isolated from normal tissue. Because the amino acid sequence of much of the molecule has been determined, it has been relatively easy to synthesize an oligonucleotide probe for screening a cDNA library prepared from mRNA extracted from the melanoma cell line.121,122 Furthermore, Pennica et al121 have cloned the entire coding sequence for PA and have engineered an *E. coli* cell to produce recombinant PA. Although PA synthesized by the melanoma cell culture method is commercially available,123 it has been estimated that recombinant PA can be synthesized at 1/200 to 1/500 the cost of the melanoma product.124 Because of the clinical potential of PA, and the difficulty incurred in purifying it from natural sources, recombinant-derived PA is likely to have little competition for a potentially expanding clinical market. Moreover, the initial results of Pennica et al122 suggest that a biologically active recombinant product can be produced by *E. coli*. Therefore, achieving the maximum level of expression in the recombinant strain should be straightforward.

**Immunoglobulin.** The plasma fractionation industry currently isolates the immunoglobulin fraction from plasma of donors hyperimmunized against known antigens. The production of immunoglobulin using methods other than plasma fractionation will probably use the cell fusion technique originally described by Kohler and Milstein.125 Although most companies are producing mouse-derived
monoclonal antibodies, their experience should prove valuable for increased human antibody production, once the techniques for the routine production of human hybridomas have been developed. Thousands of kilograms of human immunoglobulin are currently fractionated from plasma. Immunoglobulin fractionated from plasma represents a heterogeneous mixture of antibodies in which only a small percentage actually recognizes a given viral or bacterial target. In contrast, in a preparation of monoclonal antibodies, every antibody molecule reacts with the target. Thus, it should be possible to produce much smaller amounts of monoclonal antibody to replace that currently obtained from plasma.

One major problem facing monoclonal antibodies as therapeutic agents, however, will be the proof that a product derived from a cancerous cell line is free of carcinogenic agents, since it has recently been shown that some types of human leukemia result from viral infections.126

Economic Considerations

The success of modern biotechnology in producing blood products will depend ultimately upon the cost of these products relative to the cost of using plasma fractionation. Development costs (obtaining the gene sequence and then expressing the protein by recombinant cell techniques) should be far less than large-scale production and purification, where the cost of a moderate fermentation and processing facility may average 40 to 100 million dollars.126,127 Reduction in these costs may be realized by collaboration with companies that have fermentation facilities already in place.

A difficult cost to estimate is one associated with the purification of a recombinant product such that it is deemed "safe." In the case of proteins like factor VIII, where the estimated world market is only 280 g, purification should be straightforward and easily accomplished. For a product like albumin, however, where the world market is measured in tons, purification costs could be significant. Compounding that problem is the large dose of albumin that is generally given to patients, which mandates the need to completely remove any pyrogenic or antigenic contaminants from the recombinant product.

A final consideration regarding the impact of rDNA technology on the cost of plasma products involves their economic interrelationships. In the United States, the price of albumin is much higher than its production cost, because its price subsidizes the cost of factor VIII. Thus, albumin supports the plasma product market and keeps the cost of other products, like factor VIII, low. By producing factor VIII or albumin by rDNA technology, the pricing balance will be upset and it is unclear just what the price would be of products still fractionated from plasma.

CONCLUSION

With the rapid advancement of technology, blood substitutes and alternate sources of therapeutic blood components are likely to become available, although not for a number of years. Within five to eight years, at least some plasma derivatives probably will be replaced by alternate sources using recombinant DNA technology; substitutes for RBCs and platelets will be much more difficult to develop. Availability of these products will have far-reaching effects on our health care system and it is probably not too soon to anticipate the effects and plan for the consequences.

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