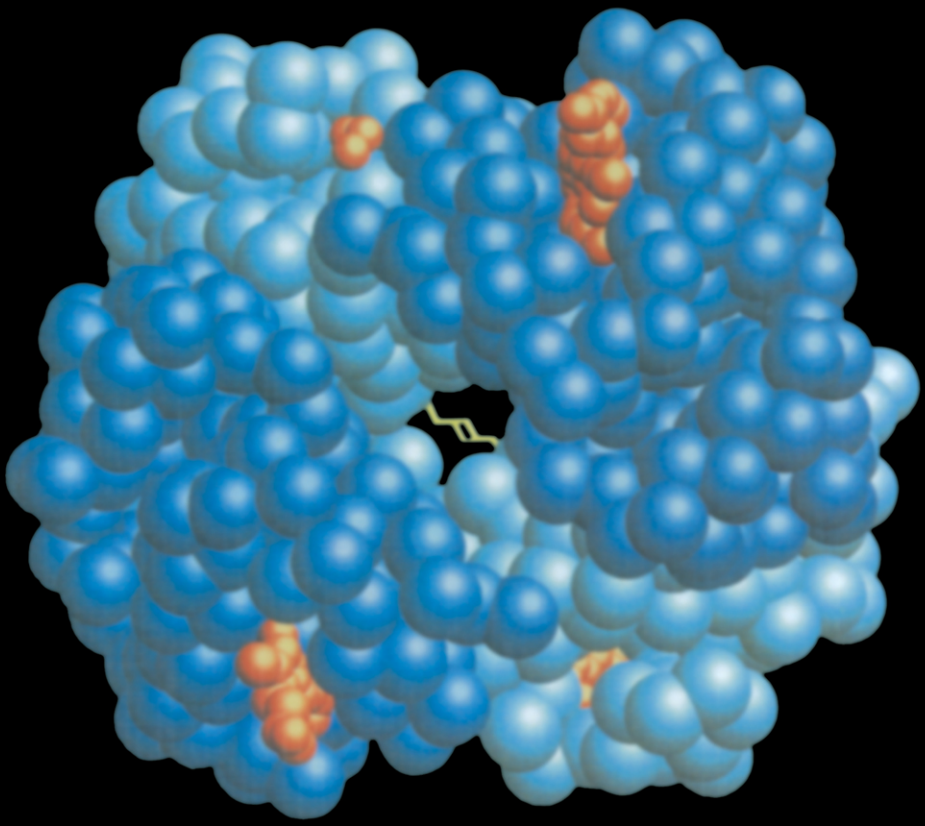
BLOOD SUBSTITUTES

Present and Future Perspectives

EISHUN TSUCHIDA, Editor



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Present and Future Perspectives

Cover illustration taken from Chapter 6 'Overview of the Effects of Diaspirin Crosslinked Hemoglobin (DCLHb) on Oxygenation, Perfusion of the Microcirculation, and Clinical Studies' by K.E. Burhop and T.H. Schmitz.

Blood Substitutes
Present and Future Perspectives

edited by

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PREFACE

This book contains the selected papers presented at the seventh International Symposium on Blood Substitutes (7th ISBS) held at Ibuka Memorial Hall, the International Conference Center of Waseda University in Tokyo on 7–10 September 1997. In keeping with the scientific design of the 7th ISBS Symposium, chapters have been carefully selected and organized to showcase the advancements in recent research. Furthermore, the newest developments after 7th ISBS are also included. The first highlight is "The Current Status of Blood Substitutes" including up-to-date clinical results of leading companies which are manufacturing hemoglobin-based or perfluorocarbon-based blood substitutes. The following chapter is the report of a roundtable discussion, "The Target and Assessment of Clinical Tests." I believe that readers will enjoy this chapter which expresses real targets of the products and issues of clinical trials, which also reflects the high activity in this area. I wish to thank Prof. M. Takaori who edited this report as a chairperson. Before the submission of the chapter, he asked all speakers to check their statements and discussions and revise them more comprehensively for readers. Indeed, the good use of hemoglobin-based materials as hemoglobin formulation is remarkable, but the development of oxygen infusion should be more important.

The following chapters are advanced techniques to evaluate the oxygen carrying efficacy in vitro and in vivo including microcirculation measurements. "Development in Encapsulated Hemoglobin," will focus on encapsulated hemoglobins as one of the prime candidates for use in the next generation of hemoglobin-based oxygen carriers. The book covers issues of hemoglobin toxicity and side effects such as vasoconstriction in more detail using carefully designed in vivo and ex vivo techniques. "Platelet Substitutes" introduce a new subject area to the session lineup at ISBS Symposia, and they are also included in this book. Of course, this book is also a collection of various new types of red cell substitutes such as recombinant hemoglobins, recombinant albumin–lipidheme complex, modified red blood cells, and perfluorochemicals using material science and molecular engineering. I hope this lineup will go a long way to make this book a successful interdisciplinary landmark toward the beginning of the coming century.

The editor wishes to thank all participants for their contributions and offers gratitude to the members of the Organizing Committee, to the Scientific Program Committee, and especially to Drs. H. Nishide and S. Takeoka, the Conference Secretariat. Finally, it is a pleasure to express indebtedness for the staff of Elsevier for their support for publishing this book and their forbearance and unfailing courtesy.

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Tokyo, May 1998

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 1

Perspectives of Blood Substitutes

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Introduction

It has been long since a number of problems were pointed out with regard to homologous blood transfusion. They include the possibilities of viral infections such as AIDS and hepatitis, antigenic sensitization such as blood group incompatibility and GVHD (Graft Versus Host Disease), various complicated tests which are time consuming and expensive, and the limitation of blood preservation (4°C, 3 weeks). However, accidents in blood transfusion cannot be avoided entirely even with close attention. Furthermore, compared with Japan, matters are more serious in many countries where the organization of blood transfusion systems are insufficient. The development of red cell substitutes is an urgent, important, and top priority subject for the lifesaving systems equipped with artificial blood (blood substitutes) that can be supplied sufficiently, safely, and instantaneously irrespective of location and blood types in case of serious accidents.

It is no exaggeration to say that the history of blood substitutes is the history of blood transfusion. Blood transfusion other than one's own blood can be regarded as the administration of blood substitutes [1]. From the middle of the 16th century, the administration of various liquids into the blood was tested, and Blundell succeeded in conducting homologous blood transfusion (1818), and proved that death from bleeding can be avoided through blood transfusion. However, the success rate of blood transfusion in those days was too low for this method to be regarded as a means of lifesaving. The success rate of blood transfusion had dramatically risen through the discovery of ABO blood typing by Landsteiner (1901), and blood transfusion as a means of medical treatment became a focus of attention. The present blood transfusion systems were established through the establishment of the blood bank (1936) in the United States and the development of a preservation method by addition of anticoagulant, ACD solution (1943). In the middle of the 1980s, the infection through blood transfusions with HIV (human immunodeficiency virus that causes AIDS) was reported, which destroyed the reliance on blood transfusion and had tremendous impact on the blood

industry, bringing about considerable reforms; the necessity for the development of blood substitutes was strongly recognized.

Requirements for red cell substitutes

Red cell substitutes must have the capability to transport sufficient oxygen to peripheral tissues; furthermore, they must keep fulfilling their functions by staying in the blood flow for an adequate period of time. Red cell substitutes are administered when more than half of all the blood is lost making it impossible to sustain life even through the administration of plasma expanders. Thus the regulation of solution properties such as viscosity and colloidal osmotic pressure is important, which will exert influence on circulatory kinetics if not adequately regulated. For example, it has been pointed out that low-viscosity preparations will cause vasoconstriction and a decrease in functional capillary density, which will decrease oxygen transport to the peripheries (Intaglietta, Chapter 10). Other opinions have been proposed: relatively high colloidal osmotic pressure will improve microhemodynamics [2]; and excessive oxygen supply will activate the autoregulation system, which will decrease oxygen supply [3]. It is thus considered necessary to prepare appropriate solution properties in accordance with adaptation with observing peripheral microcirculatory dynamics, but no practical values have been designated.

In addition, blood substitutes will not be regarded as attractive and survive in the market without effectiveness equivalent to or higher than that of homologous blood transfusion from the standpoint of safety, cost performance, and long-term preservation stability. Potentially viable preparations have been started, but will take some more time to reach completion. However, toward this purpose, several preparations have been already clinically evaluated with benefits from cutting-edge techniques, intelligence, and much support.

Properties and clinical trends of red cell substitutes

Red cell substitutes developed until now are broadly divided into two carriers (Figs. 1 and 2): hemoglobin-based oxygen carriers (1) and totally synthetic oxygen carriers (2). (1) is subdivided into acellular hemoglobin or modified hemoglobin and cellular hemoglobin or encapsulated hemoglobin; (2) is subdivided into perfluorocarbon emulsions and totally synthetic hemes. At present, modified hemoglobins and perfluorocarbon emulsions have been clinically examined in eight companies in the United States and Canada, and the others have been preclinically examined (refer to Table 1 in Chapter 2 detailing the name of the companies and types, adaptation, and the phase of clinical examination).

Acellular hemoglobin

Administration of hemoglobin, to a patient with anemia was already tried a hundred years ago by Stark [4], but this ended in failure due to serious nephrotoxicity. It was

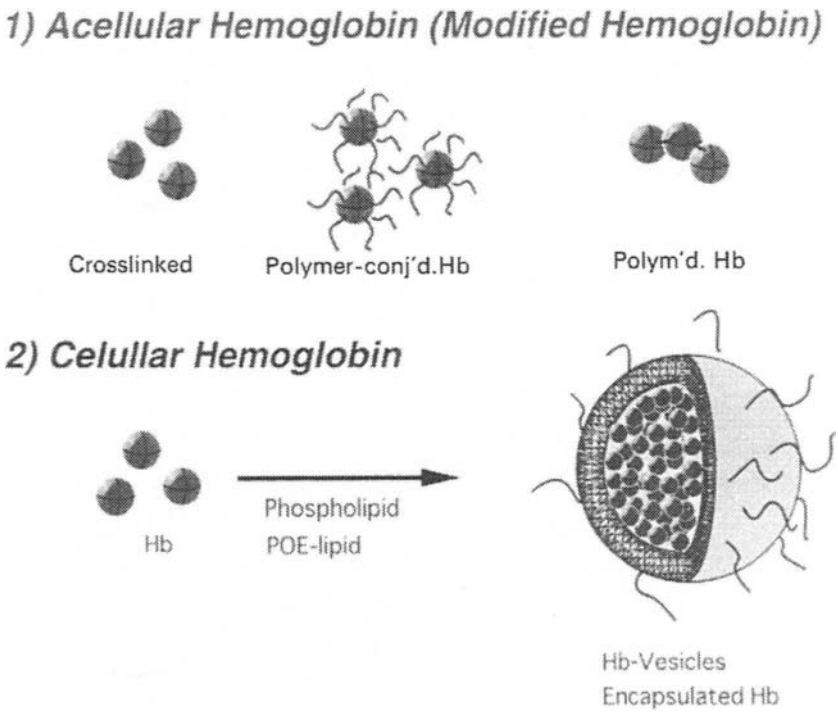

Fig. 1. Classification of Hb-based oxygen carriers.

not until the series of discoveries in 1967 that the use of hemoglobin in blood substitutes was greatly advanced. Rabinar [5] significantly decreased the side effects of hemoglobin by establishing a hemoglobin purifying method that increases the elimination rate of stroma to 99%, Bunn and Jundel [6] succeeded in considerable extension of the retention time of hemoglobin by crosslinking, and Benesch et al. [7] found the decrease in the oxygen affinity for hemoglobin combined with allosteric factors such as pyridoxal 5′-phosphate. These findings established all the basics for the present fundamental strategies for hemoglobin modification.

Acellular hemoglobin is divided into four types: crosslinked hemoglobin, polymerized hemoglobin, polymer-conjugated hemoglobin, and recombinant hemoglobin. Crosslinked hemoglobin is produced by crosslinking the amino groups of the 99th lysine between the α chains of hemoglobin using fumaric acid, which has been developed as HemeAssist^{TM} by Baxter Inc. Crosslinked hemoglobin features complete homogeneity due to its simple structure, establishment of the virus-free mass production process, an adequate oxygen affinity (33 Torr), and low viscosity [8]. However, crosslinked hemoglobin has a shorter retention time in the blood compared with the other Hb preparations, and vasoconstriction was reported as a side effect. Baxter Inc. regards the HemeAssist^{TM} as a drug in Hb therapeutics [9], and has been examining its adaptation as a blood dilutant and blood pressure stabilizer in

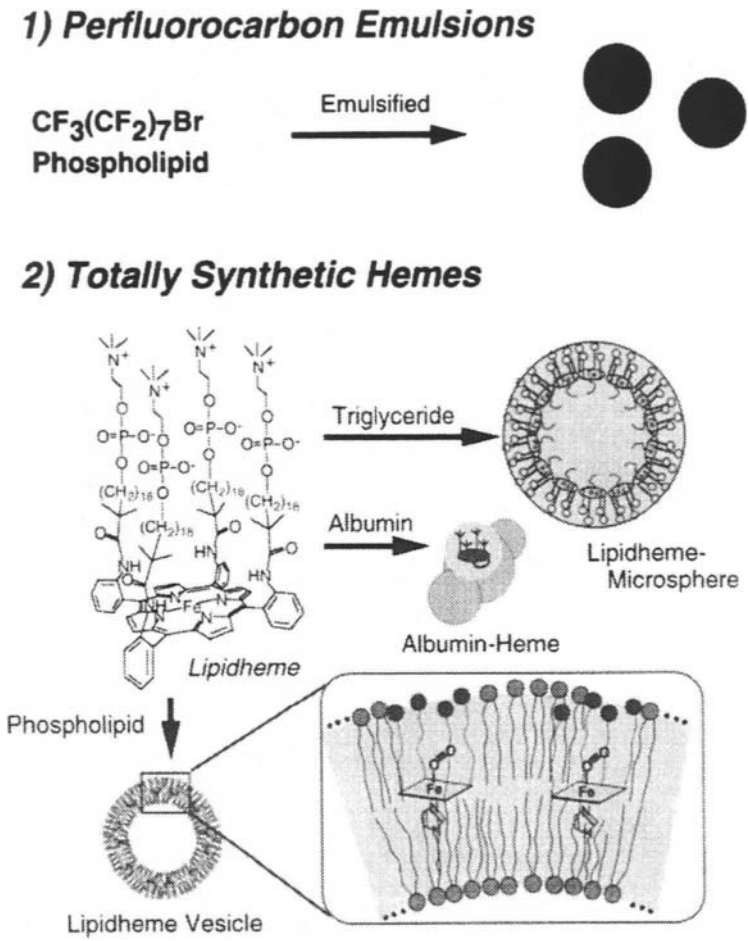

Fig. 2. Classification of totally synthetic oxygen carriers.

cerebral apoplexy, artery repair, and gastrointestinal and plastic surgery. 750 ml of HemeAssist™ was administered to patients undergoing cardiac surgery, and the necessity of additional transfusion of human red cells within 24 h after surgery examined; more than half of the patients required no blood transfusion, which was considered significantly lower than the group that was administered human red cells. Also in general surgery (aortic repair, gluteal, genucubital, abdominal, and pelvic surgery), the use of HemeAssist™ has been examined for the possibility of avoidance of transfusion of human red cells, or the decrease in transfused blood. This is detailed in Chapter 6. However, Baxter had stopped all clinical trials on HemAssist™, pending a review of clinical data showing the higher mortality than control, and shifted its efforts of blood substitutes research to the second generation of recombinant Hbs by purchasing Somatogen Inc. Baxter's attitude toward hemoglobin

therapeutics is interesting, but main target should not be changed from the replacement of blood transfusion.

With regard to polymerized hemoglobin, PolyHeme™, in which hemoglobin is intermolecularly crosslinked by glutaraldehyde, has been developed by Northfield Laboratories [10]. Hemolink™, in which hemoglobin is intermolecularly crosslinked by *o*-raffinose, has been developed by Hemosol Inc. [11]. Both of these are undergoing clinical examinations. Polymerized hemoglobins feature colloidal osmotic pressure similar to blood and extended retention time in blood, but they are heterogeneous and require the control of molecular weight distribution between 128 to 512 kD. As the clinical examination of PolyHeme™, it was administered up to six units (Hb 300 g) to patients who acutely bled from an injury or during operation, and the result was compared with that of blood transfusion (Chapter 4). As a result, no particular problems were recognized with regard to safety, and no additional blood transfusion was necessary within 24 h for more than half of the patients whose blood, the mean Hb concentration of which was 7.5 g/dl, was substituted with up to 4.8 g/dl of Hb derived from PolyHeme™. In accordance with this result, PolyHeme™ has been claimed to be effective as a blood substitute. No renal damage or fever, and interestingly no increases in blood pressure by vasoconstriction were recognized in the clinical examination of PolyHeme™, which is considered the features of hemoglobin preparations having high molecular weight. On the other hand, the increase in blood pressure was reported for Hemolink™ (Chapter 17) but small degree in comparison with crosslinked Hb, which is presumed to be due to the difference in the residual rate of low molecular weight components.

As polymer-conjugated hemoglobins, in which polyethylene glycol having low physiological activity is bound to, the surface of hemoglobin, the type using bovine hemoglobin has been developed as PEG-Hemoglobin™ by Enzon [12], and the type using human hemoglobin has been developed as PHP™ by Apex [13]. Polymer-conjugated hemoglobin features a long retention time in blood, low vasoconstriction effect, and relatively high colloidal osmotic pressure and solution viscosity, etc. Polymer-conjugated hemoglobins are heterogeneous, and are undergoing clinical tests as a tumor chemotherapy agent and a therapeutics for septic shock.

Recombinant hemoglobin produced by fungus bodies or *Escherichia coli* using transgenic techniques has been produced as Optro™ by Somatogen Inc., and is now being examined clinically in Phase II. Intermolecular crosslinking and an adequate oxygen affinity were achieved by transforming a part of amino acid sequence of human hemoglobin. The best advantage of recombinant hemoglobin is that it can be produced homogeneously and inexhaustibly in factories, but the complexity in the purification process should be resolved to establish a payable mass production process.

In Phase I of the clinical examination, a maximum of 25 g of Optro™ was administered, and no side effects such as nephrotoxicity, immune disorders, or influences on coagulation systems were recognized, but a transient increase in blood pressure up to 50 mmHg was recognized just after administration. More

than 5 g/kg of the administration caused slight dysphagia, nausea, and emesis, and deformation was recognized in the esophagus and gastrointestine [14]. A transient increase in the concentration of amylase and lipase was recognized, but no disorder was recognized in the pancreas. The aforementioned symptoms are considered to be involved with trapping of nitric oxide by acellular hemoglobin. In the first and second phases of the clinical examination, 26 g of Optro™ was administered to anesthetized patients, and the symptoms recognized in the case of volunteers in the first phase were significantly relieved. In the Phase II trial, in which Optro™ was administered up to 100 g during intraoperative blood transfusion to examine the safety and oxygen transport effect, it was reported that though a transient increase in blood pressure was recognized, no abnormality was recognized in the form of the esophagus, and no significant differences were observed in comparison with homologous blood transfusion. The application of Optro™ has been examined for acute normovolemic hemodilution in cardiac surgery and for a hematopoietic accelerating agent by the facilitation of erythropoietin generation. The recombinant preparations are detailed in Chapters 5 and 22, and their side effects are described in Chapter 9.

Cellular hemoglobin

Cellular hemoglobin or Hb vesicles have a structure in which a high concentration (> 36 g/dl) of purified hemoglobin is covered by a phospholipid bilayer membrane, which may solve the many problems in the use of hemoglobin molecules. This idea was introduced by Chang in 1957, and the development of phospholipid vesicle was advanced by the leading studies by Djordjevich and Miller [15], Hunt [16], and Farmer et al. [17] from the 1970s through the 1980s. At present, the establishment of a highly efficient manufacturing process and improvements in the properties have greatly been advanced by our group [18], and Rudolph's group [19] accumulated fundamental findings on pharmacological and physiological responses, and we have been cooperatively making preparations to advance the clinical examination.

In cellular hemoglobin, dissociation of hemoglobin tetramer to dimers is restrained because a high concentration of hemoglobin is included in Hb vesicles, and the oxygen affinity is adequately regulated and the methemoglobin formation is restrained because allosteric effectors and reduction systems are together included. The colloidal osmotic pressure is close to zero; it is thus regulatable by the addition of adequate colloids, and the viscosity can be restrained equivalent to or less than that of blood (Chapter 14). The physiological activity of hemoglobin and liberated heme can further be restrained by encapsulation into the cells. If these many advantages achieve a decrease in the cost and increase in the efficiency of capsulation process of high-purity lipid, great clinical development can be expected for cellular hemoglobin.

For example, in the examination using strips of aorta, it was proved that cellular hemoglobin showed low vasoconstriction, one hundredth of that of molecular hemoglobin. The cause of vasoconstriction by molecular hemoglobin is considered

to be trapping of nitric oxide as an endothelial derived relaxation factor (EDRF) by hemoglobin dispersed between vascular endothelium and smooth muscles. On the other hand, phospholipid vesicles cannot penetrate through blood vessel walls because they have a large particle size, 40 times of that of hemoglobin, and the binding of nitric oxide with hemoglobin is restrained due to the long mean distance from vascular endothelium because of the separating lipid membrane (Chapter 20). In addition, perfusion of acellular hemoglobin in an isolated liver causes a rapid increase in the perfusion pressure and vasoconstriction, while these symptoms are hardly recognized for cellular hemoglobin. Suematsu has pointed out that the system of trapping of carbon monoxide, which is the relaxation factor of liver vessels, is described; the sinusoid vessels of liver have holes of about 150 nm, and hemoglobin readily passes through the holes and traps carbon monoxide, which shows the significant differences between cellular and acellular structure (Chapter 19).

The results of resuscitation from hemorrhagic shock and exchange blood transfusion in the animal test using Hb vesicles are described in Chapter 14. Achievement of more than 80% of the exchange level is impossible by the administration of albumin solution alone that has no oxygen transport capacity, but all rats administered with Hb vesicles survived even with more than 90% of the exchange level. In addition, aggregation in bloodstream was effectively restrained in the system of which the surface was modified with polyethylene glycol chain, and the decrease in vascular resistance and improvement of microcirculatory dynamics were clearly recognized. A quantitative report was made on the effect of PEG chain modification on the surface of vesicles, on the basis of the measurements of the subcutaneous microcirculatory system measured by attaching a window on the dorsal skin of hamsters (Chapter 15).

Philips showed the necessity of 10 mol% PEG surface modification for the effective increase in the retention time of liposome encapsulated hemoglobin (LEH) (Chapter 12). He also observed the distribution of LEH by ^{99m}Tc labeling on the hydrophobic part of LEH lipids, and showed that 49% of administered LEH was still circulating 48 h after the administration. Szebeni (Walter Reed Army Laboratory) has been examining the influences of LEH on human complement activation to evaluate the safety of LEH [20]. The increase in C4d and Bd by the administration of LEH shows classical and the alternative pathway activation, which is due to the interaction between LEH and IgG or IgM. In addition, it was shown that IgM interacts with choline type phospholipid, and all complement activities are effectively restrained by soluble complement receptors-I (SCRI) [21]. Rudolph (Naval Research Laboratory) reported the results of the analysis focusing on the interaction between various endothelial cells and heme ingestion of acellular Hb (crosslinked Hb) and cellular Hb (LEH) in the incubation system [22]. With regard to acellular Hb, heme ingestion was particularly recognized for methemoglobin and heme oxygenase was activated with the heme ingestion, while heme was hardly ingested for cellular Hb. This is considered an advantage of cellular Hb.

Perfluorocarbon emulsions

Perfluorocarbon (PFC) solution has high oxygen solubility, but it is not miscible with water, thus PFC is emulsified with a surfactant such as phospholipid for use. PFC emulsions have several advantages such as production at a low cost, instantaneous availability in case of emergency due to long shelf stability, and no risk of infection. However, there still remain problems which must be solved, such as confirmation of the safety and metabolism, and the limit of capability including oxygen transport capacity.

The Green Cross Corp. developed the first generation of PFC emulsion, Fluosol-DA, the clinical examination of which was started in 1978; the preparation was first approved by the FDA and was introduced in the market in 1990, though the application was restricted to perfusion of colonary arteries after percutaneous transluminal angioplasty (PTCA). However, sufficient effectiveness was not obtained because of the low oxygen transporting capacity; thus its production is stopped at present.

The second generation PFCs are Oxygent™ and Oxyfluor™ developed by Alliance Pharmaceutical Corp. and HemaGen/PFC Inc., respectively, and both preparations are now under clinical examination. In the 7-ISBS session on perfluorocarbon emulsions, Riess (UCSD) and Lowe (Nottingham University), both of whom stand foremost in this field, introduced new viewpoints, in which they described the progress such as the increase in the stability of new generation PFC and in oxygen transporting capacity, the trends in clinical examinations, and various application using the properties of PFC (Chapters 7 and 25). The other PFC product; PERFTRAN, developed by Russian Academy of Science, is clinically used for patients in trauma, ischemia, transplantation, disorder in microcirculation, etc. The small particle size of 70 nm is one characteristic of this material, however, oxygen solubility of 7 vol.% under 760 mmHg pO_2 should be improved for the replacement of blood transfusion.

Totally synthetic heme oxygen carriers

In 1983, a new totally synthetic oxygen carrier that was the first in the world to use synthetic heme was developed by our group (Fig. 2). Heme is the oxygen binding site of hemoglobin, but is bound with oxygen stably as being inserted into heme pockets. In other words, each heme molecule is inserted into a specific site in the hydrophobic heme pocket structured by globin chains, by which the sixth position of heme becomes free for oxygen coordination; dimerizing oxidization is sterically prevented, and proton oxidization is prevented. This was proven by studies actively conducted in the 1970s. Besides our group, Collman, Baldwin, Traylor, Monamenteau, and others synthesized a series of derivatives to obtain such compounds that fill the aforementioned requirements [23]; only our group succeeded in the development of the system under the idea of using amphiphatic structure and molecular assembly, in which oxygen reversibly binds and dissociates in water. For about 20 years since then, we have been studying totally synthetic oxygen carriers using heme derivatives.

Besides in the system using phospholipid bilayer membrane of vesicles, oxygen transporting capacity equivalent to that of blood was found in the system using lipid heme as the surfactant of lipid microsphere, which is clinically used in a nutritional injection, in the system in which the bilayer membrane is structured by lipid heme only, in a synthesized molecule in which alkylimidazole bound with the fifth position is directly bound to the heme part, and just recently albumin-heme in which the above molecule is bound with recombinant albumin. The use of albumin that is plasma protein is expected to be safer than the use of hemoglobin.

The high efficiency of albumin-heme as an oxygen transfusion was recognized in the examination in which rats were previously treated with 70% blood drawing exchange, then were put into a shock condition by drawing a further 40% of their blood; they were subsequently administered with albumin-heme to examine its recovery effect (Chapter 24). Albumin-heme can be produced in factories because it is 100% constituted from synthetics, and has no risk of infection. It also features long shelf stability in the state of freeze-dried powder, high oxygen transporting capacity, and the capability of surface modification and introduction of reduction systems due to the molecular assembly system.

All the materials of totally synthetic oxygen carriers are inexhaustible because they can be produced in factories, but the problem of synthesizing cost still remains to be solved [24]. New ideas and compounds are continuously being proposed, and they are now being produced and evaluated.

Future prospects for blood substitutes

New development of recombinant preparations

The methodology on the production of useful proteins and enzymes using recombinant techniques will be more and more active in the future. The oxygen affinity of hemoglobin may become facultatively regulatable, and not only intramolecularly crosslinked hemoglobin but also several types of intermolecularly crosslinked hemoglobin would be obtained by the recombinant system. Somatogen Inc. has already started studying measures to resolve the problems with Optro^{TM}, that is intramolecularly crosslinked recombinant hemoglobin, one of which is the method to synthesize polymers having specific structure by crosslinking between hemoglobin molecules to extend the retention time in blood, and the other is the method to restrain the binding between heme and nitric oxide by narrowing the space of heme pockets (Chapter 5). Recently, human hemoglobin can be obtained from transgenic tobacco plants. This technique provides an inexpensive and abundant source of biomass with avoiding the risk of infection [25] Ho (Carnegi Mellon University) examined the higher order structure of hemoglobin using NMR, in which α96Val and β108Asn were substituted with other amino acids, and he described the fundamental findings involved with oxygen affinity (Chapter 22). In order to materialize recombinant preparations, those of uniform standard must be supplied efficiently and at a low cost

through the advancement in production and purification techniques. In addition, when the amino acid sequence of protein is considerably transformed from that of human Hb, it may be recognized as a foreign element in the immune system and may express teratogepicity. Therefore, long-term careful confirmation of its safety would be necessary.

Recombinant human albumin has been already developed as one of plasma substitutes by two companies, Delta Co., Ltd and Yoshitomi Phamaceutical Inc., and the reports regarding the establishment of production process of high-purity body and the results of the Phase III clinical examination were reported in 7-ISBS. Recombinant human albumin can be regarded identical to totally pure human albumin, thus the lowering of the production cost would be the only problem remaining to be solved.

New development of cellular hemoglobin

Cellular hemoglobin that can stay in blood for a long time would appear by the progress in the development of surface modifier. On the other hand, long-term maintenance of the function as an oxygen carrier requires not only the extension of the retention time in blood but also considerable restraint of methemoglobin formation. Thus, the establishment of the system is expected in which a reduction catalyst system formed by incorporating a substrate (e.g. glucose) as a reducing agent into the inner aqueous phase reduces the methemoglobin.

New types of ideas are also proposed for cellular hemoglobin. Chang (McGill University) proposed a new cellular hemoglobin, "nanocapsule", using not lipid membranes but biodegradable polylactate membranes (Chapter 13). The use of regeneration and processing of red cells would be focused on if the methods for inactivation and stable preservation of viruses are established. Fisher (Southern California University) introduced an attempt to enable the administration of red cells to anyone by modifying the surface of red cells with PEG chains to mask the blood type (Chapter 23). It would take a long time for the accomplishment, but this attempt is expected for the applications such as rejections caused by chronic blood transfusion and treatments of chronic diseases such as sickle cell anemia. Yonetani (University of Pennsylvania) suggested that α-nitrosyl HbA in which two molecules of nitric oxide are bound to α chains of hemoglobin has a considerably decreased oxygen affinity, thus it effectively transports oxygen in peripheral tissues. At present, the effects are being discussed of the system that has increased oxygen transporting efficiency by binding nitric oxide to red cell hemoglobin.

Moreover, if the biosynthesis of red cells are made possible by the culture and cloning of hematopoietic stem cells, these red cells would be used as red cell substitute having completed functions and no risk of infection. In this case, the blood type will be O type or not be expressed. Although these substitutes are not suitable for the use in case of emergency, they would be very effective for the use in waiting operations and patients with chronic anemia.

Establishment of the evaluation system for efficacy and safety

The noninvasive microhemodynamics measurements for subcutaneous microcirculation is significantly important for instantaneous direct evaluation of administration effects of drugs, etc. In this method, which was established by Intaglietta (UCSD) [26], variations in vessel diameter and blood flow velocity are measured on their images, and bloodstream is calculated from vessel diameter and flow velocity; functional capillary density is calculated for vessels in the selected field and from the number of capillaries through which blood cells pass within the time specified. Oxygen partial pressure in subcutaneous capillaries and in local tissues are noninvasively measured from the phosphorescence lifetime of the probe dependent on oxygen concentration. These measurements clarify the influences of oxygen affinity, and solution viscosity of oxygen carriers on oxygen transportation to peripheral tissues. It was concluded from this method that red cell substitutes ought to have relatively high viscosity and colloidal osmotic pressure. It is also interesting that these conclusions challenge conventional remarks (Chapter 10).

In addition, physiological behaviors and physicochemical properties of oxygen carriers are intermixed in the case of in vivo measurement, thus quantitative measurement and evaluation are generally accompanied by difficulties. Hellums (Rice University) kinetically analyzed oxygen distribution by passing oxygen carriers through oxygen permeable capillary models of 25 or 10 μm in diameter, for the purpose of the in vitro simulation of oxygen transport by a modified Hb solution to tissues (Chapter 11) [27]. In comparison with red cells, rapid oxygen release was quantitatively indicated, and definite differences between cellular and acellular Hb were revealed. This result would be important on the grounds of the opinion that excessive oxygen transport would cause vasoconstriction due to the autoregulation function.

Although the hemoglobin molecule is a water soluble protein, it is not a plasma protein such as albumin but a protein included in cells. Considering the high physiological activity and oxygen transporting property of hemoglobin, with regard to mammals, hemoglobin may be a protein that should be included in cells. In actuality, enzyme systems exist in red cells, which eliminate harmful active oxygen generated from hemoglobin and reduce oxidized hemoglobin, and in case of hemolysis, hemoglobin transfused into plasma is rapidly excluded from the plasma and metabolized by the systems with haptoglobin, albumin, etc. At present no problematic side effects or toxicity have been reported for the results of clinical examination, but increases in blood pressure, effects of Hb as an oxidant, interaction with lipopolysaccharide, complementary activity, and induction of cytokine have been increasingly pointed out (Chapter 16), thus detailed examination will be successively required for the elucidation and fundamental resolution of the functional systems.

Hereafter, biogenic reactions involved with oxygen carriers including kinetics and roles of blood will be further clarified through progress with the accumulation and interpretation of findings obtained by in vitro and in vivo evaluations, as well as findings regarding in vivo macro and micro kinetics. In addition, selective

administration of appropriate oxygen carriers in accordance with the indication will be made possible. With regard to safety, many findings have been accumulated, but many questions still remain.

Trends in artificial platelet study

The following is the summary of the study on the development of artificial platelets at a developing stage. Artificial platelets are broadly divided into two types: platelets which receive treatment for long-term preservation, and platelet substitutes having partly platelet functions, and studies have been conducted individually. Particularly the latter includes infusible platelet membrane (IPM^{TM}), thrombosphere, and thrombored cells. Refer to Chapter 27 for details, in which Alving described the status and future prospects for artificial platelets. In addition, the importance of establishing evaluation methods has been pointed out for the development of artificial platelets. Ikebuchi (Hokkaido Red Cross Blood Center) reported in the 7-ISBS session on artificial platelets that cultured human megakaryocyte as the precursor cell of platelets contained lots of GPIIb/IIIa, a protein bound to platelets, and described the possibility of mass culture. Nishiya (Keio University) reported on the development of the vesicles that effectively form compounds with platelets through surface modification of phospholipid vesicles with tripeptide (RGD) that specifically interacts with platelets. Ikeda and Murata (Keio University) proposed phospholipid vesicles introduced with recombinant GPIb α (Chapter 28), and these vesicles were shown to interact with vWF that specifically binds to platelets under coexistence of ristocetin, which can be regarded as the result that will lead the practical development of artificial platelets.

Epilogue

International conferences on blood substitutes are held every other year, which offer a venue for the exchange of information and presentation of the latest study trends in each institute involved in the field. In the seventh International Symposium on Blood Substitute held in September 1997 in Tokyo, the present research of blood substitutes was fully covered, and the status and unsettled problems of therapies using substitutes were presented in intensive exchanges between researchers and clinicians. The conference also studied the future of this indispensable research field.

In the United States, research project grants were awarded from The National Heart, Lung, and Blood Institute of NIH (NHIBI) for five years from 1993 through 1997, and the results were reported every spring as the Current Issues in Blood Substitute Research and Development Course in University of California, San Diego.

In Japan, the Society of Blood Substitutes, Japan, was established in 1993, which is actively working for the development of this field through the publication of journals "Artificial Blood", and annual conferences. Moreover, as the project to advance front line medical research, from 1997 the Ministry of Health and Welfare started the

development project of artificial blood primarily focusing on the three substitutes, artificial red cells having oxygen transport functions, artificial platelets having hemostatic effects, and artificial globulin having immune functions (Fig. 3). Also in Brazil or Korea, national projects of a considerable scale have already started.

In particular, when considering blood transfusion systems from the global viewpoint, the lack and risk of blood transfusion is incomparably higher in developing countries compared with advanced countries. For this reason, demands for the development of safe, effective, and low cost blood substitutes are increasing. The materialization of blood substitutes that has no risk of infection or side effects during transfusion and is preservable for long periods would contribute not only to the advancement in blood projects and remedies in advanced countries but also to advances in medical and welfare activities in countries and areas where medical systems are yet to be organized. Moreover its repercussive effects would be immeasurable, such as innovations in remedies for cancer, strokes, and myocardial infarction.

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 2

The Role of Blood Substitutes in Emerging Healthcare Systems

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Abstract — In the US and western Europe, the risk of transmission of viral illnesses by transfused blood has essentially reached zero. This has been achieved at great cost, however, by implementation of rigorous exclusion of high-risk donors, extensive serological testing, and reduction or elimination of unnecessary transfusions. Molecular amplification (PCR) techniques are being used for some pooled products and may be used in single blood units in certain high-risk areas. These steps have also increased the cost of blood and blood products and reduced the size of the donor pool. In most of the developing countries of the world, serious problems reduce the potential to achieve this same level of blood safety. These problems include limited economic resources to implement sophisticated testing, smaller donor pools because of endemic diseases, shortages of technically-trained personnel, and lack of a blood-banking infrastructure. Thus, it seems unlikely that all of the methods used to achieve zero-risk blood in developed countries will be successful in the developing world.

Current and future "blood substitute" products may have their most significant impact in developing countries if they are cost effective and can be adapted to local medical practice. In contrast to the developed world, where blood substitutes may be used primarily as adjuncts to elective surgery/hemodilution, the need in developing countries is for products that can be infused in severe hemorrhage, particularly in surgical or obstetrical settings. The ability to render cell-free oxygen carriers virus-free by current sterilization or purification procedures would allow even units contaminated with hepatitis virus or HIV to be processed into safe hemoglobin-based products that can be stored and used as needed. Limitations of these new products including duration of action and side effects must be clearly delineated, and their properties must be matched to clinical indications. Decisions as to the use of blood substitute products should be made at the level of national blood policies in individual countries.

Introduction

Tragically, the HIV epidemic has claimed the lives of many innocent recipients of blood products. Since 1984, aggressive testing of donor blood units, improved transfusion practices and donor self-exclusion have all but eliminated the risk in the developed countries of the world [1]. The only significant risk of HIV infection remaining in the US is a result of the HIV "window" — the period between viral infection and the detection of virus antibody in the blood — of about 22 d. A new industry has emerged, however, in the wake of that tragedy, aimed at the development

of products that can be used instead of blood and other blood-derived plasma expanders such as albumin. Ideally, such "blood substitutes" would carry oxygen, provide rapid expansion of the blood volume after hemorrhage, not require cross-matching, and would be available for immediate use.

At least nine products are currently in various stages of clinical development (Table 1) and it is possible that some of them may be available for clinicians and patients by the end of the decade.

These oxygen carriers represent a very exciting new class of therapeutics. They offer the possibility to transport oxygen to hypoxic tissues in patients with circulatory or metabolic problems, and in emergencies where blood is not available. Furthermore, they offer the hope that the huge populations of the world for which blood services are not available may have access for the first time to surgical procedures that heretofore have been dependent on transfused blood. These include, for example, transplantation procedures and surgeries that require cardiopulmonary bypass.

Table 1

Blood substitute products in clinical trials — 1997

Product (Manufacturer)	Composition	Indications	Clinical Trial Phase (1997)
PHP (Apex Bioscience)	Pyridoxylated human Hb conjugated to polyoxyethylene	Septic shock	I/II
HemAssist™ (Baxter Healthcare)	Human Hb internally cross-linked with bis(3,5-dibromo-salicyl)fumarate (DBBF)	Trauma Hemodilution	III* III*
Hemopure™ (Biopure)	Glutaraldehyde-polymerized bovine Hb	Hemodilution Sickle cell disease	IIB I/II
PEG-hemoglobin (Enzon)	Bovine Hb conjugated to polyethylene glycol	Radiosensitization of solid tumors	Ia
Hemolink™ (Hemosol)	*o*-Raffinose-polymerized human Hb	Trauma Hemodilution	II II
PolyHeme™ (Northfield Laboratories)	Glutaraldehyde-polymerized, pyridoxylated human Hb	Trauma Surgery	II III
Optro™ (Somatogen)	Recombinant di-alpha human Hb	Hemodilution Erythhropoiesis	II I/II
Oxygent™ (Alliance Pharmaceutical)	Emulsified perflubron	Hemodilution Cardiopulmonary bypass	II II
Oxyfluor™ (HemaGen/PFC)	Emulsified perfluorodichlorooctane	Cardiopulmonary bypass	II

* Clinical trials cancelled, 1998.

Is there a global shortage of blood for transfusion? No certain answer to this question is possible, but if medical practice were uniform world-wide, and if the US rate of transfusion (0.05 units *per capita* per year) is extended to the world's population of 5,863,072,031, a total annual demand for 293,000,000 units of red blood cells can be projected. No firm estimate of the number of units actually transfused is available, but Tomasulo has speculated that the number could be as low as 90,000,000 [2]. If this is estimation is accurate, it suggests that the world-wide shortfall is potentially 200,000,000 units per year. Note that this assumes a uniformly sophisticated level of healthcare, which is certainly not the case presently.

Many of the current products can fulfill essential functions of transfused blood: they can provide expansion of the plasma space and they can transport oxygen. Furthermore, development of these products has revealed unexpected properties of cell-free oxygen carriers, such as vasoactivity and autoregulation of oxygen supply [3]. While these discoveries are very exciting, they have slowed clinical development and required new regulatory procedures. Understanding their physiological effects will surely lead to new and improved products in the future. Whether or not blood substitute products will be successful in developing countries with serious, and possibly insoluble, problems of their blood supply, will depend on a number of factors including local patterns of medical practice but, most importantly, on the willingness and commitment of local governments to improve the state of the public health.

Blood availability and safety in the US and developed countries

Achievement of an essentially "zero-risk" blood supply in the US and other developed countries is now a reality [4] and is the result of efforts in several areas:

1. Avoidance of unnecessary use of blood products.
2. Improved donor questioning of risk behavior and self-exclusion.
3. Increased sensitivity of existing serological assays.
4. Development of new assays for agents of hepatitis and AIDS.
5. Development of molecular amplification to narrow window periods (PCR).

The most effective of these steps have been implementation of widespread screening for hepatitis and HIV markers and voluntary self-exclusion of donors on the basis of high-risk behavior. Current estimations of the risk of transmission of viral disease are shown in Table 2. These figures do not include, of course, the risk of clerical error leading to incorrect crossmatching, febrile, hemolytic or delayed transfusion reactions, or immune suppression. The cost of achieving this level of safety is high: HIV antibody testing has prevented 1568 new cases of HIV infection in the US at a cost of US $3,600 per quality-adjusted life year (QUALY). However, addition of p24 antigen testing would prevent only an additional 8 cases, at a cost of $60,000,000 or US $2,300,000 per QUALY. Addition of PCR testing would prevent 16 cases at a cost estimated to be US $96,000,000 or US $2,000,000 per QUALY [5]. Along with

Table 2

Risk of volunteer blood in the US [1]

Virus	Risk/unit	Confidence Interval
HIV 1/2	1:676,000*	1:202,000–1:278,000
HTLV I/II	1:641,000	1:256,000–1:2,000,000
HCV	1:125,000**	1:256,000–1:288,000
HBV	1:66,000	1:21,000–1:226,000

*Using HIV p24 antigen.
**Using anti-HCV 3.0.

increased testing, US physicians have gradually reduced the amount of blood used in surgical procedures by a variety of means, so that the overall rate of blood use was 3.1% less in 1992 compared to 1989, and the rate of autologous donations increased by 70% [6]. Whether or not blood substitutes can contribute to safe medical practices in developing countries depends on how well or how soon those countries can implement similar changes in transfusion practices.

Blood availability and safety in the "developing" world

Limited donor pools

Lack of adequate donor pools limits transfusions in many countries. For example, in Mexico, surgery is frequently delayed or canceled because of blood shortages. When blood is used, the incidence of transmitted disease, such as hepatitis, is high. Actual figures are not available, but the Director of the Cardiology Hospital of the Seguro Social, Mexico City, estimates that only 40,000 units of packed red cells are used per year in the healthcare system that cares for 80% of the Mexican population (Dr. Ruben Argüero, personal communication). Using a US and Western European transfusion rate of approximately 0.05 units *per capita* per year, Mexico's population of 90,000,000 should require 3,600,000 transfusions per year for equivalent medical practice. In Mexico, blood cannot be purchased or sold, and there is very high use in non-surgical indications such as gastrointestinal bleeding, resulting from esophageal varices in patients with cirrhosis and chronic hepatitis. At present, approximately 1,000,000 units of gelatin are used in Mexican operating rooms per year because of its low cost. This product is no longer used in the US because of questionable efficacy and because of its proclivity to cause allergic reactions. Perioperative hemodilution and priming bypass pumps with an appropriate blood substitute would be strategies whereby blood could be conserved for optimal use, and exposure of patients to risk could be minimized.

Table 3

Percentage of respondents answering correctly in Greece [3]

	Total Sample %	Sex		Age			
		male %	female %	18–24 %	25–34 %	35–44 %	45–54 %
Can you catch AIDS by giving blood?							
1987 survey ($n = 3484$)	43.0	43.8	42.1	49.1	54.8	43.5	32.5
1994 survey ($n = 1995$)	57.9	61.4	54.5	56.6	62.0	60.8	51.3
Is donated blood screened?	70.3	71.7	68.8	72.1	71.1	73.8	63.0
Is equipment disposable?	76.5	78.0	75.0	75.1	80.8	80.1	68.9

In Viet Nam, the blood supply is dependent on commercial blood donors, many of whom are high-risk drug users. A recent study found that 9% of individuals without liver disease in Ho Chi Minh tested positive for hepatitis C [7]. Furthermore, hepatitis B surface antigen (HBsAg) and anti-HBsAg were frequent in both Ho Chi Minh and Hanoi (10–14% and 35–37%, respectively), increasing in parallel with age. Anti-HCV was particularly prevalent among drug users in Hanoi (31%).

Other problems also limit the size of donor pools. Greece is typical of many non-US countries in which most blood donations are not from volunteers but rather from mandatory donations by patients' family members, i.e., "replacement" units. Two surveys in 1987 and 1994 [8] illustrate problems of perception among potential Greek blood donors (Table 3). The primary question asked was "Do you believe that one can catch AIDS by donating blood?" The percentage of correct answers in all age groups improved in the 7 years between the two studies but still was only just over 50% in 1994. In the second survey, two additional questions were added to the study, "Do you believe that blood donations are tested for dangerous infections (such as hepatitis and AIDS)?" and "Do you believe that the equipment used in blood transfusion is disposable (used only once) or not?" For each question, the most important factor determining a correct response was clearly age (poorer knowledge among older respondents) and donation history (better knowledge among those who have donated more than 3 times). Donors who had given blood only in the Army or for family credit were no better informed than non-donors. Thus while efforts to educate the public appear to be effective, progress is slow, and it is unlikely that an all-volunteer blood donor system will develop quickly.

Endemic diseases

High prevalence of endemic disease both limits the donor pool size and increases the risk of transfusion-transmitted infections. The prevalence of gastrointestinal diseases

markedly differs among developing countries because of often poor sanitation, hygiene, impure water and food ingestion, illiteracy and poverty. Gastrointestinal bleeding, diarrhea, cholera, typhoid, dysentery and viral hepatitis (A and B) can be attributed to the excessive use of alcohol, tobacco, and nonsteroidal anti-inflammatory agents. According to one recent public health analysis in India, significant reductions in liver disease would result from reductions in blood transfusions and improvements in blood banks [9]. In many underdeveloped countries, "replacement units" (i.e., blood donated by family or friends prior to surgery) or blood used urgently or in emergencies is not tested at all prior to transfusion.

The prevalence of HIV infection in the general population and donor blood pool of Thailand has increased dramatically since 1987 [10]. The HIV seropositive rate increased from 0.0065% in 1987 to 0.95% in 1993, mainly because of heterosexual transmission. In Thailand HIV antibody testing of all donor units by the National Blood Center began in 1987 and was compulsory in 1989. Donor self-exclusion began in 1991 but has not been fully effective because of limitations in donor education level [11]. An increasing prevalence of HIV infection in Thailand raises the probability of "window" transmission in that country [12] and has recently led to HIV antigen testing.

Medical care in Asia is developing rapidly. In Taiwan, the rate of bone marrow transplantation equals that of the US [13]. However, in contrast to other areas of the world, the prevalence of hepatitis B virus (HBV) is greater than 90%, and 2% of bone marrow transplant patients die of HBV-related complications. A program of immunization for HBV has been instituted because of the high prevalence of this disease. Prior history of blood transfusion is the greatest risk factor among patients with chronic hepatitis C (43.5%) [14]. This is a very serious problem, since HCV infection accounts for the bulk of post-transfusion hepatitis morbidity. In Western Europe, only 0.4–1.5% of blood donors test positive for HCV, but the disease is debilitating: 50–80% of cases lead to progressive chronic hepatitis, which leads to cirrhosis in 20–50% of patients in 10–20 years [15].

The prevalence of hepatitis is still very high in other areas of the world as well. For example, in one recent study, 14.6% of post-bypass patients in India who received blood transfusions developed hepatitis, in most cases icteric hepatitis B. All of the donor units had tested negative for HbsAg, but in retrospect, 11/48 of the units tested by PCR were positive for hepatitis B. It was concluded that serological screening was not sufficient to ensure safety [16].

African children are particularly vulnerable to transfusion-associated HIV infection. McFarland et al. [17] found that 19–47% of children hospitalized in Africa are transfused during admission, probably because of the high incidence of sickle-cell disease, malnutrition, hookworm, and other anemias. In Kinshasa, Zaire, up to 60% of HIV-infected children over the age of 2 years probably acquired the virus from a transfusion [18].

The outlook for control of transfusion-associated HIV transmission in Africa is not encouraging. In some populations of Africa, HIV seropositivity approaches 30%

Table 4

Window period blood donations [16]

Country	Donor population, year	Estimated rate of window period donations
Zambia	Monze, 1995	1 per 94 donations
Cote d'Ivoire	repeat donors, Abidjan, 1993	1 per 917 donations
Namibia	national, 1993	1 per 1527 donations
Thailand	national, 1993	1 per 4242 donations
South Africa	national, 1994	1 per 45,455 donations
United States	national (19 regions), 1995	1 per 360,000 donations

and in some areas where the prevalence has been low, such as Nigeria, seroprevalence among donor units has increased [19]. Such high prevalences will virtually assure that some true HIV-positive units will test HIV negative, increase the chance for human error and will result in the transfusion of contaminated blood (false negativity rates are low in the US but have not been confirmed in the developing world). Since the probability of such donations is dependent on the duration of the HIV window and the rate of new infections, it is likely that a significant number of donors of units that are HIV infected but that have not yet expressed HIV antibodies is significant. Indeed, the highest rates of window-period donations in the world have been reported from sub-Saharan Africa (Table 4). It is tragically ironic that molecular amplification (PCR) testing would be most effective in areas which can least afford the cost.

Transfusion practices

Blood transfusion is, in the best of circumstances, an art not amenable to rigid, clear-cut criteria. In many countries, the use of blood and blood products is part of the local medical lore. Local medical customs also influence blood use. In Africa, obstetric hemorrhage represents a large need for blood and blood products. In one study in rural Africa, <25% of all deliveries took place in hospital, including <5% of placenta praevia cases [20]. In the Cameroon, ectopic pregnancy represents an incidence of 11 cases per 1000 pregnancies, and the main complication is severe hemorrhage (45.1%) [21]. The frequency of Caesarian section rose in one hospital in rural Zaire from 6.2% in 1971 to 12% in 1992, carrying an overall risk of dying 13 times higher than for women who are able to deliver vaginally. Most of these Caesarian sections are done under emergency conditions and about 4% of them require blood transfusion [22]. A high incidence of endemic iron deficiency anemia in Mali (36.8%) was such that 2.4% of women require blood transfusion post-partum [23].

Possibilities for change

How likely is it that the developing world will adopt the practices that the developed countries have utilized to achieve a near-zero risk blood supply? The use of blood and blood products in the developed countries has probably reached a practical minimum by lowering the "transfusion trigger", the use of cell savers, autologous predeposit, and acute normovolemic hemodilution. In the developed world, about one-third of all blood transfusions are given under somewhat controlled conditions (i.e., not in emergencies) such as chronic anemia, cancer and elective surgery. Some further reductions can probably be achieved in controlled surgical settings involving cardiopulmonary bypass [24], but it is unlikely that permissible hematocrits can be allowed to be lower than current standards. In the less developed countries, the indications for transfusion are often more urgent, and even in the US the use of blood in severe or rapid hemorrhage is unlikely to diminish [25].

Serological tests being developed in the US for hepatitis and AIDS may not be totally appropriate in other parts of the world. For example, HCV genotypes 1, 2, and 3 are responsible for most cases of chronic hepatitis C in developing countries, whereas types 4, 5, and 6 are involved in other areas [26]. Patterns of transmission, especially related to drug abuse and sexual behavior, are well known in the US and Western Europe, but too few data are available for the rest of the world to determine whether risks are the same and whether the same types of screening procedures would be appropriate. Sophisticated serological testing or molecular amplification tests may not be appropriate in emerging counties because of the high cost and the expertise needed to interpret the results and to deal with the consequences of positivity. In one study in India, it was found that dried blood samples could be sent by mail to a central facility for testing for hepatitis and HIV, but that the sensitivity was reduced and results delayed [27].

Efforts to reduce transfusion-associated HIV infection in Sub-Saharan Africa by the techniques found to be effective in developed countries (HIV antibody screening, reduction of blood product use, and high-risk donor exclusion) have not been uniformly successful elsewhere. The nation-wide rate of transfusion complications in Zaire is 16% compared to 2% in Europe [28]. A review of the current status of HIV infection in Africa by McFarland et al. [17] points out that the proportion of existing and new HIV infections attributable to transfusion in sub-Saharan Africa is estimated at 10%, or in excess of 1 million cases. In most areas of Africa, a disproportionate number of blood transfusions are given to women and children, and as many as 25% of HIV-infected women and children received HIV from transfused blood.

The cost of improving blood services

The cost of improving the quality of the blood supply is a vexing and complicated issue. From a medical standpoint, physicians tend to believe that any procedure that reduces morbidity or mortality is justified, but that is increasingly not the case either

in the developed or developing world. For example, solvent-detergent treatment of plasma has been shown to be effective in eliminating enveloped virus contamination. In a recent analysis of the cost impact of this treatment, AuBuchon and Birkmeyer [29] concluded that compared with untreated plasma, a unit of treated plasma produces a net benefit of 35 min of quality-adjusted life-years at a cost of $42.5 million, or $289,300 per quality-adjusted life-year saved. These authors conclude that from a public health perspective, the relatively high cost and small benefit of reducing enveloped virus contamination with this procedure do not appear to be justified.

Few analyses of the cost of improving the safety of the blood supply have been carried out in developing countries. In one hospital in Zambia, seroprevalence for HIV among blood donors was 15.9% [30]. This study concluded that financial benefits exceed costs by a factor of 2.7–3.5. In 1991, 1073 transfusions were given, and an estimated 150 cases of transfusion-related AIDS were prevented by screening, of which 59% were in children aged 5 years or under and 31% in women. The total cost of HIV screening was $4745, and the cost per case of HIV infection prevented was $31.62. The cost of this service to the population served by the hospital was $0.03 per person. An estimated 3625 healthy years of life were saved, of which nearly 69% were children under 6, at a cost of $1.32 per year of life saved. While these figures seem compelling, the cost of a single HIV serological test (US $1–3) exceeds the *per capita* health expenditures of many African countries [17]. Additional costs associated with providing HIV-free blood would include purchasing and maintaining laboratory equipment, training personnel and providing appropriate HIV counseling.

The indications for the use of blood are not rigidly defined and vary widely from region to region, further complicating the analysis of the costs and benefits of improving the quality of blood. That is, if the safety of blood was not an issue, it is likely that more blood would be used in surgical procedures, and that more surgical procedures would be carried out. A German analysis [31] concluded that the cost of ensuring a zero-risk blood supply *in a developed country* is so large as to interfere with progress in other vital areas of health care. This raises ethical questions in regard to optimal use of available funds. In the case of blood substitutes, a definitive cost/benefit analysis cannot be done until the indications for their use are defined, and more clinical experience has accumulated to understand their potential risks and benefits.

Cultural, political and emotional issues surrounding blood

The AIDS epidemic has changed the public's perception of the safety of blood donation, as well as transfusion. In fact, the epidemic has probably reduced the overall public perception of blood banks as a community resource, introducing suspicion and fear into a relationship which the public once held above question. Increasingly, patients scheduled for elective surgery demand autologous predeposit, even when the procedure is not in their best interest [32]. Furthermore, one of the

most effective measures to reduce the risk of transfusion-transmitted HIV infection has been exclusion of donors in high-risk groups such as intravenous drug users and those with certain sexual behaviors. Identification of such high-risk donors entails a level of questioning which many potential donors may feel is an invasion of their privacy, sometimes discouraging individuals from donating blood.

Exclusion of blood donors on the basis of questions about risk factors for HIV infection is probably not as reliable in some developing countries as it is in the US and industrialized world. In one survey in Siriraj, 11.8% of women with no history of promiscuity, intravenous drug use or blood transfusions within 8 years tested positive for HIV-1 [33]. When these cases were studied more carefully in retrospect, it was determined that many of the women were concerned about their husbands' sexual or drug behavior, but were hesitant to declare their concerns. Hence, cultural forces seem to have prevented these women from being assertive enough in their relationships to reduce the incidence of sexually transmitted disease.

In many countries where paid donation is illegal, obligatory donation of blood by friends and relatives of a patient is routine. When a patient is admitted to hospital for a surgical procedure, it is required that he/she provide a specified number of units of donor blood. Contrary to the intent, this practice encourages donations from unqualified persons, because not to fulfill the requirement could mean denial of medical care to a friend or relative. In Mexico, the blood very often does not reach the blood bank reserves and is used, instead, in emergencies such as acute gastrointestinal hemorrhage. In Russia, another country where blood cannot be bought or sold, donors receive free transportation to collection centers or paid absences from their jobs. Again, this practice encourages high-risk donors who are most in need of payment.

Blood substitutes products

At least nine products are currently in clinical trials (Table 1). These products fall into three general classes of blood substitutes: those based on hemoglobin, those based on perfluorocarbons, and liposome-encapsulated hemoglobin. The latter group of products, liposomes, are perhaps most like native red blood cells, but the cost and complexity of manufacture have slowed clinical development. No liposome-hemoglobin products are currently in human clinical trials. Perfluorocarbon emulsions, while inexpensive and not limited by supply, require supplemental oxygen to be administered in order to be effective, and the maximal doses that can be given are not yet clear.

Classes of hemoglobin-based blood substitutes

Products based on hemoglobin would seem to be the best candidates for application in the developing world, and they fall into 3 general subtypes: crosslinked hemoglobin

Table 5

Classes of hemoglobin-based blood substitutes

Class	Examples (see Table 1)	Intravascular persistence (h)	Oncotic pressure	Viscosity	Vasoactivity
Crosslinked hemoglobin tetramers	HemAssist™ Optro™	~12	low	low	marked
Polymerized hemoglobin tetramers	HemoPure™ HemoLink™ PolyHeme™	~12–24	low	low	moderate
Surface-modified hemoglobin tetramers	PHP PEG-hemoglobin	~24–48	moderate–high	moderate–high	mild

tetramers, polymerized tetramers, and surface-modified hemoglobin (Table 5). For a detailed description of the specific chemical modifications of various products the reader is referred to Winslow [34].

Intramolecular crosslinking of hemoglobin by any of a number of chemical means produces a molecule that is cleared from the circulation more slowly than native hemoglobin and has reduced renal toxicity. An example is human hemoglobin crosslinked between the α chains with bis(3,5-dibromosalicyl)fumarate (DBBF). The product has been called $\alpha\alpha$-hemoglobin by the US Army [35], DCLHb and HemAssist™ by Baxter. Optro™, a genetically crosslinked hemoglobin manufactured by Somatogen, contains a further mutation that reduces its oxygen affinity so that its oxygen binding curve is similar to that of normal human blood. Intramolecularly crosslinked hemoglobins tend to have relatively short intravascular retention times, low oncotic pressure and viscosity, and significant vasopressor activity.

Polymerized hemoglobins are intermolecularly cross-linked. Glutaraldehyde was one of the first polymerizing agents to be tested extensively in blood substitute development [36]. Northfield Laboratories' product, PolyHeme™, is glutaraldehyde-polymerized human hemoglobin obtained from outdated blood. Similar products, based either on bovine hemoglobin (Hemopure™, Biopure Corp.) or human hemoglobin (HemoLink™, Hemosol, Ltd.) are also being developed commercially. Polymerized hemoglobins have many properties that are similar to those of human blood, including oxygen affinity, oncotic pressure and viscosity. They have somewhat longer intravascular persistence and are slightly less vasoactive, but the polymerization process is an inherently less controllable reaction, and post-modification processing may need to be more extensive.

Surface-modified hemoglobins take advantage of the 42 lysine residues on the

surface of the molecule, which present free amino groups for potential modification. Various derivatives of polyethylene glycol (PEG) have been reacted at these sites to produce large molecules which are relatively low in antigenicity and persist for prolonged periods in the circulation. One such product, human hemoglobin modified with PEG, was developed by Ajinomoto and Apex Bioscience. Since the hemoglobin is also reacted with pyridoxal phosphate prior to reaction with PEG, it has acquired the working name PHP (pyridoxalated hemoglobin polyoxyethylene). A similar product, PEG-hemoglobin, is manufactured from bovine hemoglobin by reaction with PEG. Unique features of this class of hemoglobins are their high oncotic pressure and viscosity.

Potential applications for blood substitutes

Initial interest in blood substitutes by military physicians was because of their potential for treatment of trauma victims. The US Army established that $\alpha\alpha$-hemoglobin produces significant systemic and pulmonary hypertension in some animal species and subsequently abandoned its efforts to continue development. Baxter continued development of this product, supporting its efforts with the viewpoint that pressure elevations in shock will improve tissue perfusion, and that the vasoactivity of its product will reduce the dose required for a therapeutic effect in hypotensive patients. Clinical trials for Polyheme™ are underway in trauma patients, and it is too soon to know how successful they will be. Promising results have been reported using Optro™ in intraoperative surgical blood loss. Surgical hemorrhage is a potentially more controlled clinical testing than trauma.

Hemodilution refers to the practice of removing a portion of a patient's red blood cells and replacing them with a plasma expander, either a colloid or a crystalloid, prior to surgery. The practice, more common in Europe than in the United States, has the advantages that the patient's own red cells can be set aside for later infusion instead of allogeneic blood. Alliance Pharmaceutical Corp. has proposed this use for its oxygen carrier, Oxygent™, and a number of manufacturers of hemoglobin-based products, including Somatogen, Hemosol, Biopure, and Baxter, have also explored this application.

Cardiopulmonary bypass is being used increasingly worldwide as coronary artery bypass procedures become more generally available. Each procedure requires that the bypass pump be primed with some plasma expander, either blood or a colloid. If the prime is colloid, then patients usually require blood transfusion in the postoperative period. If the priming fluid is blood or red cells, then the patient must either be hemodiluted or exposed to the risk of allogeneic blood. If blood substitutes are proven to be safe and effective, they may be the ideal solution for pump priming and could enable countless surgical procedures in the developing world.

Many other clinical applications are being explored. For example, Optro™ has been used in patients with "end stage renal disease" and in patients with refractory

anemia in conjunction with exogenous erythropoietin. The combination appears to act synergistically to stimulate erythropoiesis [37].

Limitations of blood substitutes

Products that use outdated human blood as their source will be limited by availability of outdated units or collection of blood from donors. Many of the manufacturing processes lead to overall yields of only 50%, in which case collection will have to exceed product by a factor of two. This could be problematic, because blood donation is often perceived as a charitable act, and donors might not be as willing if they know their blood is to be used to manufacture product for profit. If paid donors are used, the risk of contamination will increase. Animal blood will raise the concern of transmission of animal pathogens. For example, while the risk of transmission of the Bovine Spongiform Encephalitis (BSE) virus may be very remote, it is perceived as a great danger, especially in Europe and Canada [38]. Recombinant hemoglobin may be the ultimate hope, but so far the process is limited by low yields, high cost, and the necessity to purify hemoglobin from bacterial components of fermentation.

The side effects of the various products are still not completely understood. Chief among these are gastrointestinal complaints and vasoconstriction in the case of the hemoglobin products. As clinical development continues, additional information will become available, such as whether anesthetized patients exhibit the same side effects or whether new ones arise as interactions with anesthetics are observed.

A common problem among all blood substitutes is their short duration of action. The plasma residence times for hemoglobin-based products range from approximately 12 h for cross-linked hemoglobin to about 2 d for PEG-hemoglobin. These times are very short compared to a mean residence time of 120 d for a human red blood cell. The hope, however, is that the new products will be used in clinical situations in which banked blood can be eliminated or reduced, such as in surgical bleeding or hemodilution. None of the current products would be optimal in patients with chronic anemia.

Development of blood substitutes has also raised new issues with regard to mechanisms of oxygen transport. When an oxygen carrier is present outside of the red blood cell, the diffusion of oxygen to tissues is facilitated [39,40,41]. At first consideration, this might seem to be an advantage. However, consider that the mammalian circulation is "engineered" around cellular oxygen delivery, and has sophisticated mechanisms in place to regulate the amount of oxygen that reaches tissues (autoregulation). These mechanisms have evolved to ensure maximal oxygen delivery in anemia or hypoxia. Autoregulation is a complex mechanism which increases blood flow locally to tissue to compensate for too little oxygen, but also limits flow when an excess of oxygen is sensed. Understanding of this mechanism has been facilitated by the study of cell-free oxygen carriers [42].

The cost of the new products could be a significant impediment to their widespread use. Companies developing them are not yet in a position to project ultimate cost,

because manufacturing scale, source of raw materials, and exact procedures are not yet established. However, it would appear that costs of products in the range of the current cost of banked human blood will be necessary in order for products to be competitive, unless significant advantages over blood can be demonstrated.

In developing countries, the cost issue is complex. First, it is difficult to ascertain exactly what the costs of current transfusion practices are in many countries because transfusions are usually provided as a part of a socialized medical system. Second, transfusions are often provided in "tiers" — that is, blood obtained from family members, private donors, or hospitals could all have different prices (and safety standards). Finally, the most complex issue is what impact safe transfusion practices would have on the overall economy of the healthcare system. Any definitive analysis must consider the impact not only of caring for post-transfusion complications, such as hepatitis and HIV infection, but also of caring for patients who are denied surgical procedures. For example, what is the relative cost to support a patient who is disabled and unable to work because of degenerative or other joint disease compared to the cost of a joint replacement by elective surgery and returning the patient to work?

Regulatory issues

Procedures for regulatory approval of new medical products vary widely among developing countries. In addition to approval on the basis of safety and efficacy (usually according to US FDA standards), new products usually need to be "registered". This often involves clinical trials to be carried out locally, even if extensive data are already available. In addition, many countries have centralized purchasing for government-sponsored healthcare systems, which means that price and supply must be negotiated on a competitive basis at annual or semi-annual intervals. In any case, approval by the US FDA or its equivalent is probably a prerequisite to large-scale introduction of a blood substitute product in most developing countries.

There is still not a clear agreement on the demonstration of efficacy of blood substitutes. The recognition that cell-free hemoglobin differs from blood in many respects has led to a refinement of expectations for regulatory approval. The US FDA requires that safety and efficacy of new therapeutic agents be established [43]. Demonstration of the efficacy of blood transfusions would be difficult, and comparison of new products which can perform only some of the functions of blood will be still more problematic. Most of the potential end points would be very difficult to demonstrate for blood transfusions, not to mention "blood substitutes". For example, trials designed to demonstrate improved symptoms, better end-organ function, or tissue oxygenation for patients who receive blood substitutes compared to blood would be exceedingly difficult and would undoubtedly be massive and expensive. Shortened hospitalization and fewer postoperative complications, such as infections or myocardial infarction, for example, may be quantifiable end points, but

would still be very difficult. Perhaps reduced use of allogeneic blood transfusion is the most likely end point and amenable to quantification in the short term. In regard to the developing countries, this is the most significant end-point.

Conclusions

Achievement of "zero-risk" blood for the world as a whole using methods that have been successful in the developed world is an unrealistic goal. The primary reasons are that the steps needed to ensure total safety of the blood supply are too expensive for most of the world, endemic diseases are too prevalent to eliminate infected donors and the pattern of clinical indications for blood transfusions will be nearly impossible to standardize. Furthermore, certain problems which are not of primary concern in the US, such as bovine spongiform encephalitis virus (BSE), have recently raised fears in Europe and Canada that yet other diseases may be transmittable by blood transfusion. An obvious solution to this situation is the introduction of safe, low-cost "blood substitutes". The likelihood that blood substitutes can be successfully introduced into a developing health care system will depend on the degree to which a given product meets local needs and can contribute to the overall economy and priorities of healthcare. Developers need to be aware of these local factors.

Implementation of blood substitute programs in developing countries should provide significant reductions in transmission of viral diseases in high prevalence areas if products can be made to be safe and available at reasonable cost.

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 3

Red Cell Substitutes: Evolution of Approaches for Demonstrating Efficacy

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Introduction

Regulatory agencies, such as the US Food and Drug Administration (FDA), are charged with ensuring that drugs and biologics are safe and effective. In the case of red cell substitutes, many safety issues have been resolved, but the demonstration of efficacy has been, and continues to be, a special challenge for both regulators and for investigators. In this discussion, we will review the approaches taken in attempts to demonstrate efficacy and will consider the effect of these approaches on the progress toward an approved product.

Background

The definition of criteria for demonstrating the efficacy of red cell substitutes has been a problem from the beginning of research on these materials. This problem derives, in large part, from the fact that red cells, *per se*, have never been subjected to a controlled clinical trial for the purpose of demonstrating their efficacy. There is, therefore, no standard with which to compare the red cell substitutes. In addition, the paucity of fundamental research in the area of oxygen transport does not support use of laboratory assays as definitive endpoints. This is an important point and it would be well to consider it before going on.

A major factor in decisions made regarding criteria for demonstration of efficacy has been the FDA regulation that defines efficacy (effectiveness):

"*Effectiveness means a reasonable expectation that... pharmacological or other effects of the biological product... will serve a* clinically significant function *in the diagnosis, cure, mitigation, treatment or prevention of disease in man.*" [1]

The concept presented in this regulation helps us differentiate between *efficacy* and *activity* [2]. The definitions could be stated as follows:

Efficacy is demonstrated by production of a clinical benefit.
Activity is demonstrated by results obtained in biological or chemical or physical assays.

The FDA determined that there was a consensus of scientific opinion that supported requirement of the demonstration of *efficacy* (a clinical benefit endpoint) rather than simply of *activity* (a surrogate endpoint). This determination set the stage for issuance of formal guidance [3], which affected the clinical studies that have been done in the past few years.

We will now review in more detail the history of approaches to demonstration of efficacy and the expected and actual results of these approaches. We will conclude with some reflections on alternate outcomes or, what might have happened if....

Demonstrating efficacy: history

As noted above, the problem of demonstrating efficacy has been on the minds of investigators from the outset. It has been a special problem with red cell substitutes since the criteria for transfusing red cells constitute a subject that is, itself, mired in controversy [4]. We will concentrate on the activities since the late 1960s, when two important milestones were reached: studies on hemoglobin-based agents were stimulated by the development of stroma-free hemoglobin and it was demonstrated that perfluorocarbons could be infused as emulsions.

A chronology of some major events in the history of demonstration of efficacy of blood substitutes is presented in Table 1.

Much of the early thrust for development of red cell substitutes came from military needs, but the toxicity associated with administration of red cell hemolysates prevented any serious attempts at clinical studies. The observation by Rabiner et al. in 1967 and 1968 [5], that removal of residual red cell stroma with ultracentrifugation or filtration prevented adverse events mediated by disseminated coagulopathy led to initiation of human studies with an emphasis on use in military and civilian trauma. Episodes of renal toxicity again slowed progress until investigators recognized that hemoglobin moieties that could pass the renal glomerulus were potentially nephrotoxic.

Table 1

Demonstrating efficacy: chronology

1960s–1970s:	Anticipated uses in trauma
1980s:	Emphasis on replacing red blood cell use (AIDS)
1983:	Fluosol I. Studies in Jehovah's Witnesses
1989:	Fluosol II. Approved by the FDA for use in oxygen-carrying drug in angioplasty
1990:	FDA Points to Consider on Safety
1994:	FDA Points to Consider on Efficacy

While those working on hemoglobin-based materials concentrated on obtaining purer and appropriately modified preparations, Fluosol was infused into human subjects in Japan and clinical studies were begun in the US. These studies utilized patients who required transfusions but who refused blood for religious reasons. The early anecdotal studies seemed to suggest that the product conferred a benefit to the patients, but a controlled study did not confirm this and the product was not approved by FDA for use as a red cell substitute [6]. Fluosol was resubmitted in 1987 as an oxygen-carrying drug for use in angioplasty and was approved for this indication in 1989. The efficacy of this drug was demonstrated by cardiac function studies that were felt to adequately represent clinical benefit (satisfactory surrogate endpoints). Although this product did not succeed in the marketplace, it was the first, and thus far the only, approved therapeutic agent whose mode of action is based on facilitating oxygen transport.

By 1989, investigators felt that they had sufficiently pure materials and that the mechanism of renal toxicity was understood and controlled by appropriate hemoglobin modification. Unexpected and poorly understood adverse reactions in clinical trials were encountered.

In the late 1980s led to an NIH-FDA meeting in which active manufacturers presented their data to an advisory committee, in private and separately. Although proprietary concerns prevented data and experience from being shared, the input permitted the advisory committee to produce the Points to Consider on Safety. This guidance identified specific testing programs to be applied to all products and led to a period in which the understanding of mechanisms of toxicity increased rapidly. While these Points emphasized safety, a closing paragraph addressed efficacy:

Efficacy, as well as safety, should be demonstrated under experimental conditions that mirror the intended clinical use. Of particular importance are:
Function of proposed product in presence of RBCs;
Measurement of tissue and organ function as an indication of efficacy;
Comparison of product with efficacy of transfused RBC.

The clinical trials done in the years following issuance of this guidance utilized products that had been tested according to protocols that followed the guidance and such testing was relatively uniform from manufacturer to manufacturer. However, the testing stopped short of multiple manufacturers submitting their products for testing in a single, well described system. The US Army and the FDA issued an invitation to manufacturers to test their materials in the pig model developed at the Letterman Army Institute of Research [7], but the invitation went unheeded. This was an invitation by the FDA, not a requirement. More on that later.

Phase I and some early Phase II trials were accomplished and investigators began giving serious consideration to endpoints of pivotal clinical trials that would support approval of these products. It was clear there was not sufficient understanding of the products under development to permit use of surrogate endpoints. For example,

Table 2

Stratification of endpoints

Endpoint	Comments
Acute hemorrhagic shock	Objective endpoints (e.g., mortality rate) possible, but limited safety data delayed such trials.
Regional perfusion	Early animal and early clinical studies showed some favorable activity for oxygenating ischemic organs or enhancing radiation effects. More complex trials did not follow.
Perisurgical applications	Investigators concentrated on avoidance of red cell transfusions as the pivotal endpoint. This was the only specific example of an endpoint given by FDA. This emerged as the most popular choice among manufacturers seeking initial approval.

increased hemoglobin levels did not demonstrate a successful transfusion of a red cell substitute, as they do a transfusion of red cells, because we do not understand enough about the function of soluble hemoglobin to equate it with red cell hemoglobin. Since there were no uniformly accepted criteria that could be used to evaluate the efficacy of red cell transfusions, attention focused on the ability of red cell substitutes to permit patients to avoid red cell transfusion. While public concerns about blood product safety tended to support such study designs, the execution of studies utilizing a transfusion-avoidance endpoint was quite difficult due to the lack of uniform criteria for giving transfusions.

Once again, in 1994, the FDA sought expert advice and public input, this time regarding more detailed guidance on efficacy. After considerable discussion and reflection, the Points to Consider on Efficacy were issued [8]. The requirement for demonstration of clinical benefit did not change, but different types of clinical indications were recognized and the point made that the efficacy criteria for each would differ. The stratification of indications is shown in Table 2.

For all of these potential applications, it was the intent of those who participated in the 1994 meeting that the investigations that followed would lead to a gradual but consistent increase in the understanding of the mechanism of red cell substitute products and that this increased understanding would serve as the basis for scientifically sound clinical trial efficacy criteria. The basis of the problem was the insufficient basic research applicable to acellular oxygen carriers had been supported and accomplished in the preceding 30 years. Those were years when molecular genetic research was abundantly funded and the outcome supported the development and growth of the biotechnology industry. In addition, since the research that was done was performed in industrial laboratories, there was incomplete publication of results, especially publications describing negative experiences.

Demonstrating efficacy: expectations and realizations

When the Points to Consider on Efficacy were released, it was expected that there would be an accumulation and publication of data correlating red cell substitute use with organ and animal function. There has not been an apparent increase in publication of such pertinent data, but we have observed a number of clinical trials, many focused on elimination of red cell transfusion. We have come to understand that these trials are difficult to design and to execute. In addition, there is now an increased focus on the cost of medical care and on cost/benefit considerations. This latter element is part of the recent onslaught of managed care into the medical care-financing arena. At this time, it will be interesting to speculate on possible outcomes from policies and courses of action that were not taken.

Alternate outcomes (or, what would have happened if...)

A. Military use of an experimental product

Shortly after issuance of the Points to Consider on Safety, perhaps encouraged by the realization that the renal toxicity of hemoglobin-based products was understood, some investigators proposed using hemoglobin solutions for military casualties in Operation Desert Storm. Although there were fewer casualties than expected in this military operation and only a small percentage of the 100,000 units of frozen red cells was actually transfused, use of the products available in early 1991 may well have resulted in a large number of poorly understood adverse reactions. Such reactions, now appreciated as functions of molecular size of the product and related in part to nitric oxide interactions, at the least would have caused concern and anxiety. In the battlefield setting, this could have led to serious problems and would likely have reflected poorly on the industry, the investigators and those who permitted use of an investigational product on military personnel. In retrospect, the conservative approach taken at that time seems to have been appropriate.

B. Requirement for comparative studies with red cells

Some advisors had recommended that red cell substitutes should be tested in clinical trials in which red cells were transfused as the control. The recommendation went on to state that the results of such trials must show comparable benefits resulting from both arms of such a trial. This recommendation was never seriously entertained and it is instructive at this time to recognize the wisdom of that course of action. As noted above, there is not a consensus position regarding criteria for transfusion of red cells, nor is there agreement on endpoints that demonstrate the efficacy of red cells. Given these facts of life. The proposed trials comparing red cell substitutes with red cells would have been most difficult, if not impossible, to design and execute.

C. Some advisors suggested that all manufacturers should test their products in a set of standard models in order to obtain more meaningful safety and efficacy data

As mentioned previously, an invitation to use the Letterman pig model had been issued, but there were no responses. Subsequently, investigators have disagreed about a number of issues associated with specific products, including vasoreactivity, blood flow distribution and blood pressure effects. It is interesting to speculate whether a requirement by the FDA that all manufacturers test products in a specific model or set of models would have benefited the entire field. There would have been problems. For example, the model(s) must be chosen, the study protocols must be agreed upon and the FDA must verify that it has the authority to mandate such testing. Nonetheless, since FDA had been unable to motivate industry to use standard models voluntarily or to increase publishing and sharing of data, this might have been an appropriate move. Whether it would have been successful, whether it would have benefited the field as a whole can only be speculated upon at this time.

The Future

The difficulties encountered by investigators studying red cell substitutes are due, in large part, to the incomplete store of knowledge regarding oxygen transport, especially in the setting of acellular oxygen carriers. This research deficit has been partially corrected in recent years both by NIH and by industry. It is reasonable to assume that red cell transfusions will be studied and that outcome analyses will be done using sophisticated modern technology. It is similarly reasonable to assume that methods for the precise definition of outcome from red cell transfusions will be applied to red cell substitutes.

Conclusions

There has been considerable progress in the past decade toward development of a red cell substitute.

The industry has not been required to test products in standard models. Comparative data on existing products are very scarce.

The scientific community involved with blood substitutes, as a whole, may have benefited from comparative data on available products and the development and possible approval of products may have been expedited.

Methods for the precise definition of outcomes from red cell transfusions will be applied to red cell substitutes.

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CHAPTER 4

The Clinical Utility of Human Polymerized Hemoglobin as a Blood Substitute Following Acute Trauma and Urgent Surgery*

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Introduction

World-wide interest continues in the development of a clinically useful O_2 carrier to serve as a blood substitute [1]. Such an O_2-carrying resuscitative fluid should provide simultaneous volume expansion and O_2-carrying capacity, be universally compatible, immediately available, free of vasoactive properties, free of disease transmission, and capable of long term storage. An alternative to human blood would be particularly desirable in the setting of acute unplanned blood loss and would simplify the approach to resuscitation following hemorrhage. Previous clinical trials with dissociable tetrameric hemoglobin (Fig. 1(a)) solutions revealed unacceptable adverse effects [2–5]. These trials, however, were conducted using unmodified tetrameric human hemoglobin and consistently demonstrated the recognized toxicities of renal dysfunction and vasoconstriction. Additional adverse effects included fever, coagulation defects, hepatic dysfunction, gastrointestinal symptoms, flank pain, and other nondescript symptoms. The Savitsky study in 1978 [2] provides the most compelling data. Healthy volunteers were infused with 0.25 g/kg of unmodified tetrameric human hemoglobin with an average plasma [Hb] of 57 mg/dl. The subjects developed gross hemoglobinuria, decreased urine production and glomerular filtration, elevated blood pressure and reduced heart rate. Clearly, unmodified tetrameric hemoglobin is unsafe for human use.

The early explanations for these complications emphasized residual stromal contamination, but in recent years there have been several notable advances in our understanding [1]. The first was the development of a primate model which could

* Printed with permission from Williams & Wilkins, originally published in the Journal of Trauma 1997, 43(2): 325–332. The clinical utility of human polymerized hemoglobin as a blood substitute following acute trauma and urgent surgery. S.A. Gould, E.E. Moore, F.A. Moore et. al.

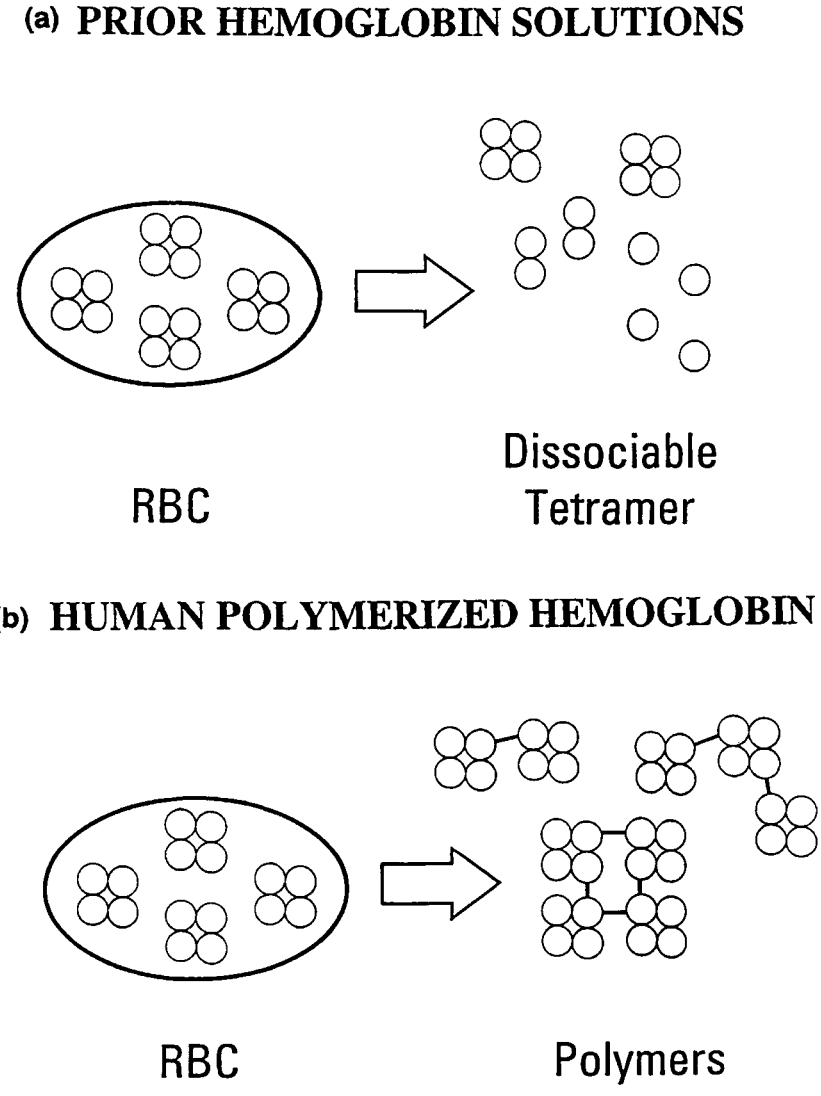

Fig. 1. (a) Preparation of unmodified, dissociable tetrameric hemoglobin from human red cells; and (b) preparation of polymerized, tetramer-free hemoglobin from human red cells.

accurately reproduce the Savitsky findings following infusion of tetrameric hemoglobin [6]. Second, as a result, was the evolution of stabilizing modifications of the hemoglobin molecule. The primate model provided a system to accurately assess the safety of each modification to the tetramer. The third was the recognition of the key role of nitric oxide, endothelial derived relaxing factor (EDRF), in the regulation of vascular tone.

Nitric oxide produced by the vascular endothelial cell is present on both the

luminal and abluminal sides of the vascular conduit. The tetramer readily binds nitric oxide within the lumen, but also extravasates through the endothelial cell wall to the vascular muscle layer, binding abluminal nitric oxide and resulting in vasoconstriction. Renal dysfunction, probably attributable to a combination of monomer and dimer filtration through the kidney with secondary mechanical tubular damage, is perhaps extended by renal vasoconstriction.

Consequently, the goals in preparing a safe modified hemoglobin solution should include preventing filtration into the kidney, as well as extravasation beyond the vascular endothelial layer. The approach used in developing human polymerized hemoglobin (Poly SFH-P) has been to create a large, iso-oncotic molecule through hemoglobin polymerization and to remove all unmodified tetramer (Fig. 1(b)). The details of the preparation and characterization of Poly SFH-P have been described previously [7,8]. We have documented the safety of a one-unit (50 g) infusion of Poly SFH-P injection in healthy volunteers [9]. The purpose of the current FDA-approved Phase 1/Phase 2 study is to begin to assess the therapeutic benefit of Poly SFH-P as a blood substitute in treating the acute blood loss that occurs following acute trauma and urgent surgery.

Materials and Methods

Description of hemoglobin solution

Poly SFH-P is a sterile, pyrogen-free, isotonic, and iso-oncotic solution. The physiologic properties are summarized in Table 1. Following lysis of human red cells, the native tetrameric hemoglobin is polymerized using glutaraldehyde [7,8]. Pyridoxal phosphate is used to obtain a p_{50} that is elevated compared to the normal red cell p_{50} of 26 Torr. Essentially all unreacted tetramer is then removed. One unit of Poly SFH-P is composed of 50 g of hemoglobin in a 500 mL solution. This is the equivalent to the mass of hemoglobin functionally delivered in a single unit red cell transfusion. Poly SFH-P has a shelf-life of at least one year.

Table 1

Poly SFH-P characteristics

Volume	500 ml
Mass Hb	50 g
[Hb]	10 g/dl
p_{50}	28–30 Torr
Met[Hb]	< 3%
Tetramer	< 1%
$T_{1/2}$	24 h
Shelf Life	≥ 1 year

Experimental protocol

This study was conducted at Denver Health Medical Center (DHMC), an American College of Surgeons certified Level I adult and pediatric trauma center. DHMC serves as the Rocky Mountain Regional Trauma Center. The protocol was reviewed and approved by the Colorado Multiple Institutional Review Board. Informed consent of the potential risks and benefits associated with this research study was obtained from either the patient or appropriate surrogate in all enrollments. The study design was a prospective, non-randomized, open-label trial. All patients were under the care of one of the authors (E.E.M., F.A.M., J.M.B.). Male and female patients of at least 18 years of age were eligible for the study. The inclusion criteria included the following:

(a) Blood loss due to acute trauma or urgent surgery;
(b) Clinical decision for urgent transfusion in anticipation of low [Hb]; and/or
(c) Systolic blood pressure < 100 mm Hg due to blood loss.

The exclusion criteria included:

(a) Severe head injury (Glasgow Coma Scale ≤ 8);
(b) Lack of acute blood loss;
(c) Signs or symptoms of pre-existing organ dysfunction;
(d) Clinically determined pregnancy (history or physical examination).

When a clinical decision was made to initiate transfusion, Poly SFH-P was infused in lieu of allogeneic blood. All infusions were supervised by the study coordinator (J.B.H.) to observe for any unexpected events in the course of the patient's therapy. The first 10 patients were eligible to receive one unit of Poly SFH-P, the next 20 patients were eligible to receive up to three units of Poly SFH-P, and the last 9 patients were eligible to receive up to six units of Poly SFH-P. The goal was adjusted as the study proceeded to identify those patients who were likely to receive the maximum allowable dose. After receiving the maximum eligible dosage of Poly SFH-P, all patients completed any further necessary transfusion therapy with red cell transfusions as indicated. Infusions were given preoperatively, intraoperatively, and postoperatively to both awake and anesthetized patients.

Measurements

Samples were obtained at pre-infusion, end of infusion, 12 h, day one, day two, and day three. Vital signs (temperature, blood pressure, heart rate) liver function (AST, ALT, bilirubin), amylase (not measured in the first 10 patients), and renal function (creatinine clearance, creatinine) were measured. Hematocrit, total hemoglobin, red cell hemoglobin, and plasma hemoglobin concentrations were determined using a Coulter JT (Hialeah, FL) and an IL 482 CO-Oximeter (Lexington, MA).

Since Poly SFH-P circulates via the plasma, oxygen transport determinations

comparing red cell and Poly SFH-P are possible. Following infusion of Poly SFH-P, arterial and venous blood samples were simultaneously obtained from a given regional perfusion bed (central or peripheral). Oxygen contents (vol% O_2) were measured on an IL 482 CO-Oximeter and a Ciba-Corning pH/Blood Gas Analyzer 170 (Medfield, MA) and are expressed as follows (eqn. (1)) for arterial samples (same for venous samples):

$$C_aO_{2,TOTAL} = C_aO_{2,RBC} + C_aO_{2,Poly\,SFH\text{-}P} + C_aO_{2,DISSOLVED}. \quad (1)$$

Percent oxygen utilization (extraction ratio) is calculated according to the following simplification of the Fick O_2 consumption/O_2 delivery relationship (eqn. (2)):

$$\%O_2\,\text{Utilization} = \frac{C_{a\text{-}v}O_{2,\,RBC\,or\,Poly\,SFH\text{-}P}}{C_aO_{2,\,RBC\,or\,Poly\,SFH\text{-}P}} \times 100. \quad (2)$$

The utilization of allogeneic blood was recorded for each patient during the initial 24 h period following their blood loss.

Statistical methods

Results were compared at each stage of the study to the pre-infusion data using a paired t-test, or an unpaired t-test for the O_2 utilization data, and statistical significance was assessed at the $p < 0.05$ level. All data is expressed as mean ± standard deviation.

Results

The 39 patients ranged in age between 19 and 83 years. The 28 male and 11 female recipients represented all racial and ethnic backgrounds. Eleven patients sustained blunt and penetrating trauma each and 17 underwent non-trauma related surgery (aortic aneurysm, hepatic resection, portosystemic shunt and hip replacement).

Poly SFH-P infusion

The infusion rate ranged from 1 unit in 175 min to 6 units in 20 min, depending on the urgency of the situation. Infusions were administered preoperatively ($n = 3$), intraoperatively ($n = 23$), and postoperatively ($n = 13$) to both awake and anesthetized patients. Fourteen patients received 1 unit of Poly SFH-P, 2 patients received 2 units, 15 patients received 3 units, and 8 patients received 6 units. Three complaints of labile blood pressure or chills were documented, but subsequently reoccurred with further administration of other blood products. There were no significant safety issues or adverse events related to the infusion of Poly SFH-P.

Hemoglobin data

The data for [Hb] was determined according to the following relationship (eqn. (3)):

$$\text{Total}\,[\text{Hb}] = \text{RBC}\,[\text{Hb}] + \text{Poly SFH-P}[\text{Hb}] \quad (3)$$

Table 2

Hemoglobin concentration (g/dl) in study patients

	RBC [Hb] (g/dl)	Poly SFH-P [Hb] (plasma) (g/dl)	Total [Hb] (g/dl)
Pre-infusion	9.7 ± 2.6	0	9.7 ± 2.6
1 Unit	8.3 ± 1.9	1.3 ± 0.5	9.3 ± 1.7
2 Unit	6.4 ± 1.2	2.5 ± 0.7	8.5 ± 1.1
3 Unit	5.5 ± 1.1	3.5 ± 0.8	8.5 ± 0.6
6 Unit	2.9 ± 1.2	4.8 ± 0.8	7.5 ± 1.2

Each 50 g, single unit infusion of Poly SFH-P raised the plasma hemoglobin approximately 1 g/dl, as shown in Table 2, maintaining total [Hb] as the RBC [Hb] fell as a result of progressive hemorrhage (Fig. 2).

Oxygen utilization

The data for O_2 loading and unloading, and percent O_2 utilization at the end of Poly SFH-P infusion are shown in Table 3. The mean RBC [Hb] was 7.2 ± 2.7 g/dl, and the plasma (Poly SFH-P) level was 2.4 ± 1.4 g/dl. The O_2 content for the arterial and venous samples, as well as the arteriovenous content difference for each carrier are presented. The O_2 utilization from Poly SFH-P Injection was increased compared to the O_2 utilization from the red cells ($p < 0.05$).

Safety

The data for temperature, mean arterial pressure (MAP), heart rate, and creatinine clearance are shown in Figs 3 and 4. There were no significant changes in any of

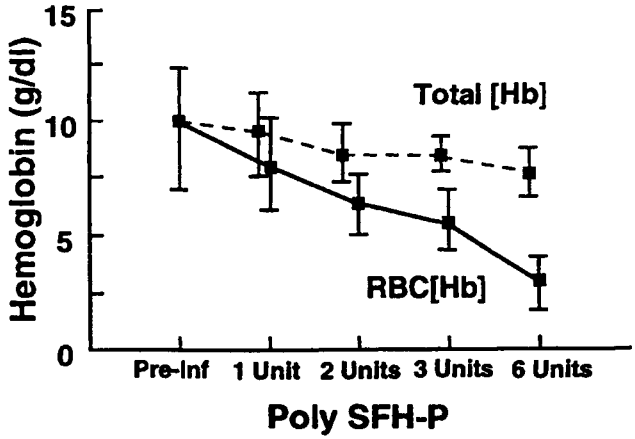

Fig. 2. Data for total [Hb] and RBC [Hb] before and after Poly SFH-P infusion.

Table 3

O_2 Loading/unloading

	Red Cell	Poly SFH-P
CaO_2 (vol%)	9.7 ± 3.6	3.2 ± 1.9
CvO_2 (vol%)	7.0 ± 3.1	2.0 ± 1.3
Ca-vO_2 (vol%)	2.7 ± 2.2	1.2 ± 0.9
O_2 Utilization (%)	27 ± 16	37 ± 13
[Hb] (g/dl)	7.2 ± 2.7	2.4 ± 1.4

these parameters, documenting the absence of any vasoactive properties of Poly SFH-P.

The data for creatinine, AST, ALT, total bilirubin, and amylase are shown in Tables 4 and 5. Table 4 shows the data for the entire population. There is a large variance in these parameters resulting from the many abnormal starting values due to the injuries. Table 5 shows the data excluding those individuals who had abnormal pre-infusion values, in order to assess the influence of Poly SFH-P in the absence of pre-existing pathology. The results indicate the lack of any change in these values following the infusion of Poly SFH-P.

Acid-base status was assessed by an end-infusion arterial blood gas: $pH = 7.36 + 0.1$ and $pCO_2 = 35.9 \pm 6.9$. Lactic acid measurements were not done.

Blood utilization

During the initial 24 h period following blood loss and Poly SFH-P infusion, 23 (59%) of the 39 patients avoided allogeneic blood exposure and were infused exclusively with Poly SFH-P as the O_2 carrier. The distribution of allogeneic transfusions during this 24 h period is illustrated in Fig. 5.

Discussion

The requirements for development of any new therapy include the documentation of safety, efficacy or physiologic activity, and patient benefit. A composite of these parameters permits an overall assessment of clinical utility. This is the first report to evaluate the clinical utility of human polymerized hemoglobin as a blood substitute in the setting of acute trauma and urgent surgery.

The safety of infusing 1 unit (50 g) of Poly SFH-P in healthy volunteers has been previously reported [9]. There was no evidence of fever, renal dysfunction, vasoconstriction, hepatic dysfunction or other adverse effects historically associated with tetrameric hemoglobin solutions [10]. Many of these effects continue to be reported

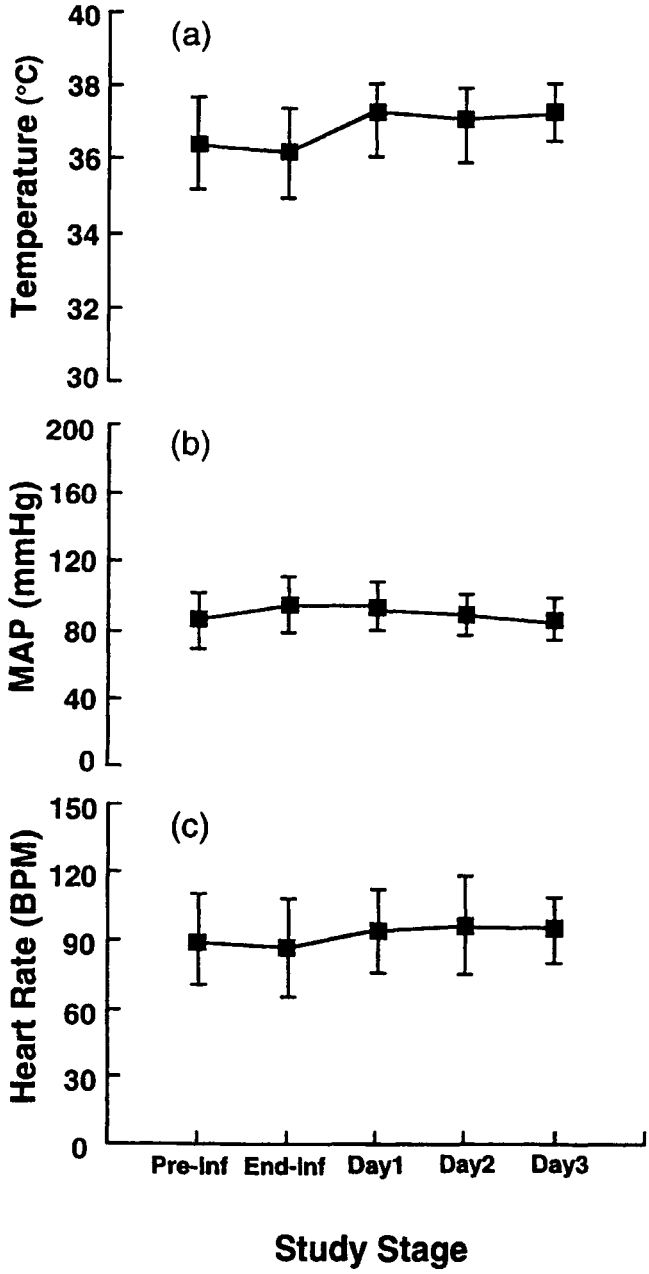

Fig. 3. (a) Data for temperature before and after Poly SFH-P infusion; (b) data for mean arterial pressure before and after Poly SFH-P infusion; and (c) data for heart rate before and after Poly SFH-P infusion.

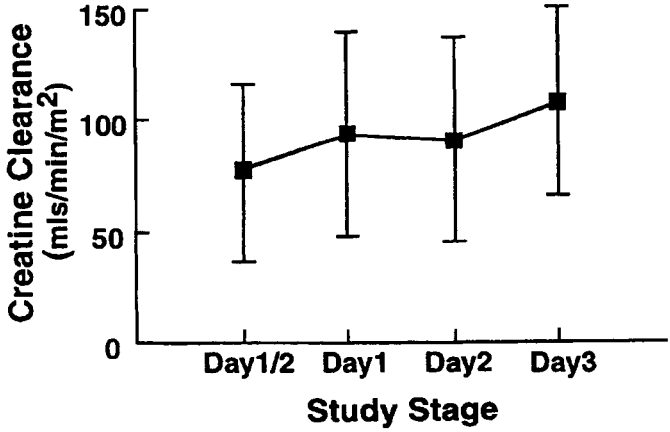

Fig. 4. Data for creatinine clearance before and after Poly SFH-P infusion.

Table 4

Organ function for the entire population

	Pre-Infusion	Day 1	Day 2	Day 3
Creatinine (mg/dl)	0.7 ± 0.3	0.8 ± 0.3	0.7 ± 0.4	0.7 ± 0.3
AST (U/L)	80 ± 99	115 ± 129	97 ± 95	71 ± 68
ALT (U/L)	75 ± 113	70 ± 94	68 ± 102	63 ± 82
Bilirubin (mg/dl)	0.9 ± 1.2	1.9 ± 2.5	2.8 ± 4.1	3.9 ± 6.5
Amylase (U/L)	48 ± 22	135 ± 192	110 ± 163	93 ± 101

Table 5

Organ function after excluding patients with abnormal preinfusion values

	Pre-Infusion	Day 1	Day 2	Day 3
Creatinine (mg/dL) ($n = 39$)	0.7 ± 0.3	0.8 ± 0.3	0.7 ± 0.4	0.7 ± 0.3
AST (U/L) ($n = 25$)	33 ± 29	60 ± 39	54 ± 42	47 ± 45
ALT (U/L) ($n = 26$)	25 ± 22	28 ± 26	26 ± 20	28 ± 23
Bilirubin (mg/dL) ($n = 26$)	0.7 ± 0.8	1.1 ± 0.9	1.6 ± 1.6	2.9 ± 5.4
Amylase (U/L) ($n = 19$)	48 ± 22	103 ± 130	97 ± 106	97 ± 91

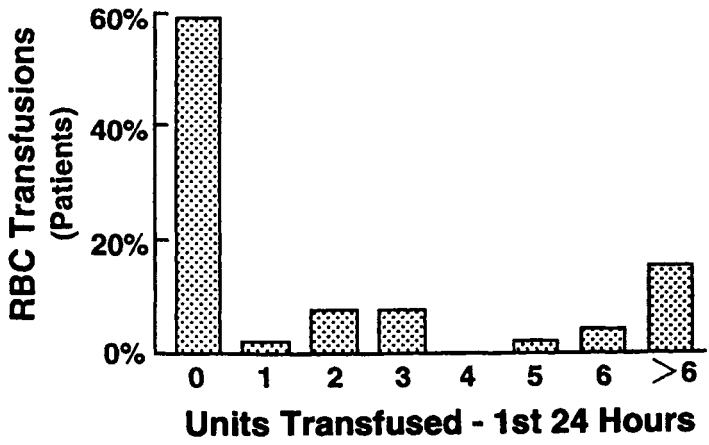

Fig. 5. Distribution of allogeneic RBC transfusions in patients who received Poly SFH-P during the first 24 h of hospitalization.

with some of the modified hemoglobin solutions currently being evaluated [11–15]. The results of this trial demonstrate that the infusion of up to 6 units (300 g) of Poly SFH-P in bleeding trauma and surgical patients is also free of adverse effects.

Figures 3 & 4 illustrate the lack of vasoconstriction, renal dysfunction, or fever, and Table 4 reveals the lack of organ dysfunction. The small rise in bilirubin on day 3 is likely due to the clearance of the circulating hemoglobin. The AST and ALT results reveal there is no evidence of hepatocellular damage. The data validate the concept that polymerization of the hemoglobin molecule and removal of unmodified tetramer will eliminate the adverse effects. These results document the apparent safety of the rapid infusion of large volumes of Poly SFH-P in acute unplanned hemorrhage.

There is no agreement at present on the proper approach to the assessment of efficacy or physiologic activity of a blood substitute [16–18]. Blood serves as a relevant standard of reference for the evaluation of efficacy of alternative O_2 carriers. Currently, the indications for transfusion differ between patients, physicians, clinical settings and institutions. Further, in the setting of acute, ongoing blood loss, most decisions to transfuse are based on clinical anticipation of the deterioration of the patient status without transfusion. In 1988, the National Institutes of Health addressed the issue of transfusion guidelines with a Consensus Conference on the peri-operative use of red cells [19]. The consensus statement reflected the contemporary standard of care that transfusions are rarely indicated with a [Hb] > 10 g/dl, and are usually indicated with a [Hb] < 7 g/dl. Although there is considerable debate regarding the optimal physiologic approach to transfusion, there is agreement that the 7–10 g/dl range, delineated by the NIH, is likely the therapeutically desirable level. Irrespective, clinical judgment of the patient status remains the most important element in the transfusion decision. The assessment of efficacy is based on the

presence of an adequate hemoglobin concentration, physiological evidence of utilization of the delivered oxygen, and the absence of clinically evident ischemia [20–25].

This clinical trial of Poly SFH-P as an O_2 carrying resuscitative fluid has adopted a similar approach to the assessment of efficacy. The consented bleeding patient was assessed for transfusion needs according to traditional practice. If transfused, the patient received Poly SFH-P as indicated; between the minimum 1 unit (50 g) dose to 6 units (300 g) as their oxygen carrier. Oxygen loading is predicated upon a functionally sufficient supply of circulating hemoglobin, assuming the process of oxygenation is adequate in the lungs. The ability to load and unload oxygen is a fundamental requirement of an O_2-carrying resuscitative fluid, which can be quantitatively described by the arterial oxygen content (loading), and the arteriovenous oxygen content difference (unloading). The relationship between oxygen loading and unloading is expressed as the percent oxygen utilization (extraction ratio) [20–25]. Since the Poly SFH-P, in plasma, is easily separated from the red cell component of whole blood by centrifugation, the hemoglobin determinations and oxygen contents of the Poly SFH-P can therefore, be quantified precisely, and compared to those of the red cells [26].

The physiologic effect of the infusion of each unit of Poly SFH-P is demonstrated in Table 2. The increase of approximately 1 g/dl per 50 g unit of infused Poly SFH-P is similar to the 1 g/dl [Hb] increase noted following a single unit red cell transfusion. The ability of Poly SFH-P to maintain adequate [Hb] despite the progressive fall in the red cell [Hb] during ongoing hemorrhage is illustrated in Fig. 2. The pre-infusion total hemoglobin concentration of 9.7 ± 2.6 g/dl is consistent with the transfusion threshold of 7–10 g/dl in the NIH consensus statement [19]. According to the study protocol, the patients would receive Poly SFH-P while bleeding progressively up to 6 units of blood without receiving red cell transfusions.

In addition to documenting the presence of Poly SFH-P in the plasma, the physiologic efficacy was functionally evaluated by comparing oxygen loading and unloading of the Poly SFH-P to that of the circulating red cells. Global O_2 transport interpretations cannot be made in this study since most of the blood samples obtained were taken from a peripheral vein. However, a valid assessment of the relative performance of Poly SFH-P and red cells in a given regional perfusion bed is achievable. Table 3 shows in vivo and in vitro Poly SFH-P equivalence to red cells in terms of O_2 loading, unloading and utilization. The O_2 utilization from the Poly SFH-P is higher than from the red cells ($p < 0.05$), consistent with the increase in p_{50} and consequent rightward shift in the oxyhemoglobin dissociation curve. The most relevant observation is the evidence that the hemoglobin carried by the Poly SFH-P is functional physiologically.

The final consideration is the benefit to the patient. By infusing an adequate amount of physiologically active Poly SFH-P in lieu of allogeneic red cells, it should be possible to reduce exposures to allogeneic blood in the scenario of acute hemorrhage. In a previous review we found that the majority of trauma patients (59%) transfused within the first 24 h following acute blood loss received 1 or 2 units

of blood [27]. In retrospect, it may be argued that some of those transfusions were not necessary. Although both blood and blood substitutes should be used appropriately, the use of a universally compatible, disease-free, temporary O_2-carrying resuscitative fluid should be helpful in this setting. Figure 5 illustrates the distribution of allogeneic red cell utilization during the initial 24 h post-injury or post-surgery period for the 39 study patients. In this trial, 23 (59%) of the 39 patients who received Poly SFH-P as their oxygen carrier avoided allogeneic transfusion during the initial bleeding period following infusion of Poly SFH-P. Since each of these patients would have otherwise received blood, the availability of Poly SFH-P reduces the need for allogeneic blood during this period.

In summary, this report illustrates the ability of Poly SFH-P to safely and adequately maintain circulating hemoglobin levels at a therapeutic level without the infusion of red cells following acute blood loss, while effectively loading and unloading oxygen. This protocol tested Poly SFH-P in the most relevant setting of urgent hemorrhage and suggested a benefit to the patient by reducing the need for allogeneic transfusion therapy. Although this was a non-randomized open-label study, the results document the initial evidence of clinical utility of Poly SFH-P as a blood substitute in this setting.

There is potential for such a therapy to significantly alter the early resuscitation regimen. Figure 6 reveals the volume and red cell loss that occur as a result of hemorrhage of 30% of the normal blood volume. Current resuscitation involves

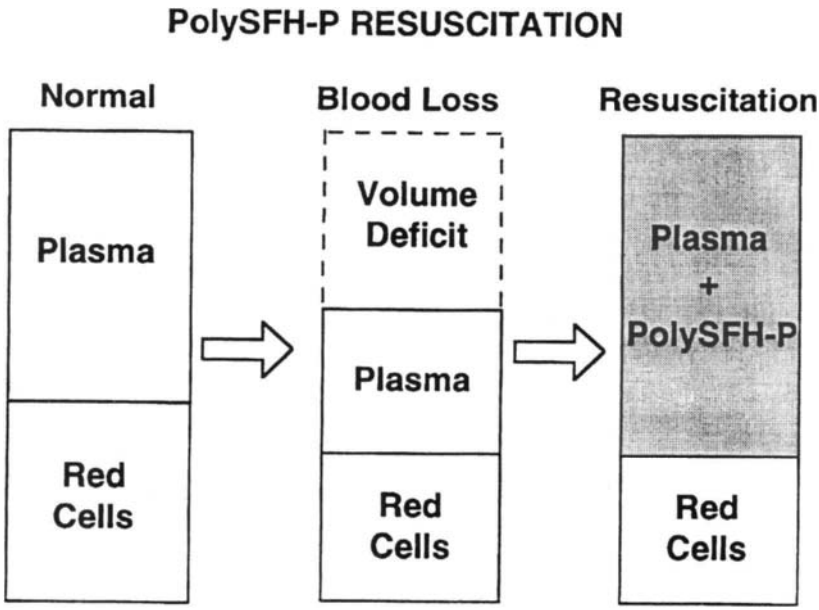

Fig. 6. Illustration of the normal blood volume, the changes following a 30% blood volume hemorrhage, and the restoration following resuscitation with Poly SFH-P.

initial asanguineous volume replacement, followed by red cell transfusions when compatible blood is available and necessary. A universally compatible, safe O_2-carrying resuscitative fluid would permit simultaneous and rapid volume expansion and hemoglobin replacement without allogeneic red cell transfusion (Fig. 6). In addition to simplifying the initial approach in the emergency department, such therapy could be extended to the pre-hospital setting where blood is not available. Randomized and controlled studies are currently underway [28] to further establish efficacy and more precisely determine the eventual role for Poly SFH-P in the care of the severely injured patient.

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 5

Recent Progress in the Development of Recombinant Human Hemoglobin (rHb1.1) as an Oxygen Therapeutic

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Introduction

Tissue oxygenation

Maintaining a stable level of oxygenation in all tissues, but especially those vital to life, is an essential requirement for normal physiological function. In fact, vital organs begin to shut down and die within minutes of a disruption in oxygen delivery. To protect against such a catastrophic outcome, key organs are fed by several primary and collateral blood vessels; circulating red blood cells normally exist in over abundance; shunting and changes in vascular tone can redirect blood flow to areas of need. Despite these safeguards, vital organs are susceptible to a sudden reduction in oxygen delivery, resulting in acute cellular hypoxia which is a leading cause of death.

Organs and tissues become hypoxic when insufficient oxygen is delivered to the cells to support metabolic needs. Tissue hypoxia is defined as a condition in which cells of a tissue have abnormal oxygen utilization such that the tissue is experiencing anaerobic metabolism [1]. Hypoxia may result if red cells are lost or prevented from reaching organs. Usually, this occurs one of two ways: red blood cells are decreased by serious bleeding, often due to trauma, surgery, or some other cause of bleeding, or red cells cannot reach vital organs due to blockage of a major blood vessel, as occurs during a stroke or heart attack.

Bleeding. Serious blood loss is treated by transfusing red blood cells collected from donors. A blood transfusion provides volume and most importantly, oxygen delivering capacity. Donor blood must be typed and crossmatched to ensure compatibility with the recipient and a mistake in testing can result in death. In situations of trauma, the time required for matching donor blood with the recipient can mean the difference between life and death. Blood transfusions administered in association with surgery are performed to prevent tissue hypoxia from occurring. Exposure to donor blood, however, carries with it a finite risk of transmission of infectious diseases (such as AIDS and hepatitis) and complications of transfusion-related immunosuppression

[2,3]. Concerns about transfusions with donated blood have created a significant need for minimizing its use.

Ischemia. Both chronic and acute blockage of arteries is the cause of myocardial and cerebral ischemia and hypoxia. Therapy is aimed at removing the cause of the blockage (e.g. thrombus or embolus) and reestablishing flow. In the area of coronary artery blockage, several options exist. Thrombolytic agents are employed as well as several surgical techniques [4]. For cerebral blockage (stroke) there are fewer choices. Surgery is usually not an option and thrombolytics have just started to be cautiously used for some types of stroke. Despite great strides in the treatment of both myocardial and cerebral ischemia, the acute hypoxia still leads in many cases to organ damage and even death. Time is of the essence to reverse the hypoxia, and other therapeutic agents that could minimize the impact of the lack of oxygen are needed.

Tissue oxygenating therapeutics

Hemoglobin solutions possess characteristics that make them well-suited for acute oxygen delivery. The hemoglobin molecule is much smaller than a red blood cell. Hemoglobin solutions might be able to travel past blockages and even through blood clots to reach vital organs. Also, by not having to squeeze through capillaries (where oxygen diffusion to tissue occurs) in a manner similar to red blood cells, hemoglobin solutions are more efficient at delivering oxygen than a red blood cell. Hemoglobin solutions may be useful for preventing or reversing acute hypoxia and preserving vital organ function until more definitive medical or surgical intervention can be applied.

There have been numerous attempts to develop a hemoglobin-based oxygen carrying solution for use in medicine, most particularly as a therapeutic which can be used in surgery as a temporary oxygenating agent to minimize exposure to donor blood. Many of these activities are ongoing today and involve hemoglobin extracted from human or bovine red blood cells (stroma-free hemoglobin). However, stroma-free hemoglobin has several characteristics which must be corrected in order to make it suitable for use as a therapeutic agent. First, when human hemoglobin is removed from the human red blood cell, its oxygen affinity increases, thereby causing the hemoglobin to bind oxygen too tightly and significantly reducing oxygen delivery to the tissues [5]. In order for human-derived stroma-free hemoglobin to be used as an effective oxygen therapeutic, its oxygen affinity must be reduced by chemical treatment [6]. Such modification, however, is not required for bovine-derived hemoglobin [7]. Second, stroma-free hemoglobin tends to dissociate (or separate) into two alpha-beta globin pairs (called "dimers") which can be toxic to the kidneys [8]. Dissociation can be avoided in stroma-free hemoglobin by chemically linking, polymerizing or otherwise chemically modifying the hemoglobin molecule [9,10].

Recombinant hemoglobin technology

Somatogen has successfully cloned, expressed and purified recombinant human hemoglobin (rHb1.1, trademarked Optro®) that binds oxygen in a manner similar to hemoglobin contained in human red blood cells [11]. rHb1.1 is a molecular variant of human hemoglobin (Fig. 1) wherein, (1) the two alpha globin chains are fused with a glycine bridge between the C-terminal amino acid of one alpha globin chain and the N-terminal amino acid of the other alpha globin chain, and (2) the amino acid located in position 108 of both beta globin chains has been changed from a lysine to an asparagine.

The fusion of the two alpha globin chains prevents dimerization, and the single amino acid substitution at position 108 in the beta globin chain (the same change found in naturally occurring Hb Presbyterian) corrects the oxygen affinity to allow efficient oxygen delivery ($p_{50} = 33$ mmHg at 37°C). X-ray crystallographic studies of the deoxy form of rHb1.1 at 2 angstroms resolution revealed that deoxy-rHb1.1

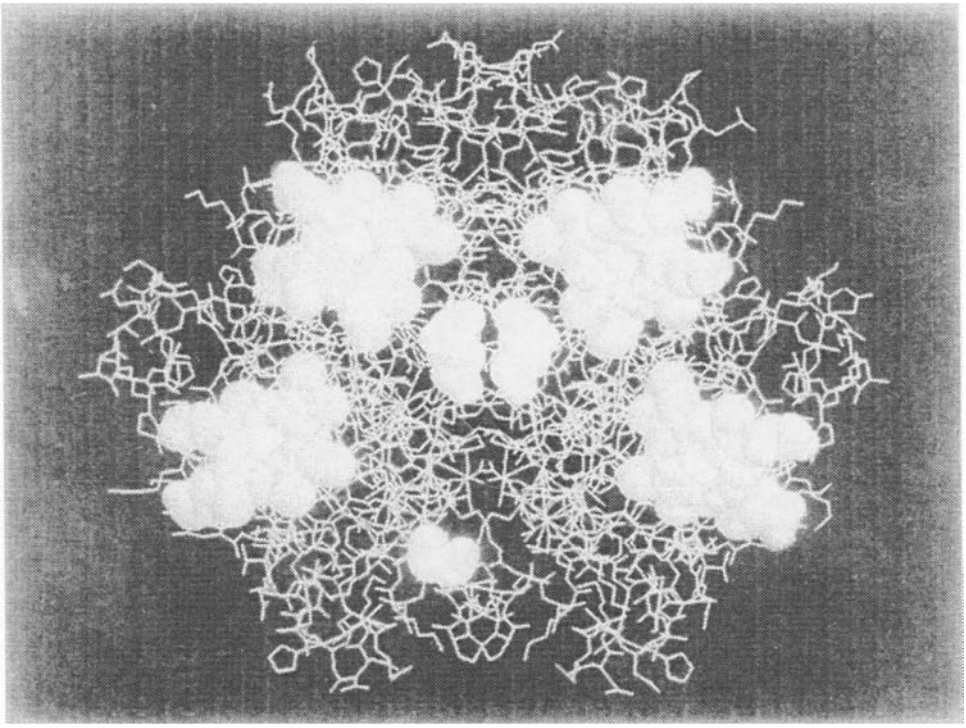

Fig. 1. 3-Dimensional structure of rHb1.1. Heme moieties are identified with space filling atoms in each of the four subunit domains. Presbyterian mutations (β-108-asparagine substitution) are identified with space filling atoms in the center of the molecule. The glycine bridge between the two alpha chains is found at the bottom of the molecule.

displays the "T-state" quaternary structure very similar to the structure of deoxy native hemoglobin [12].

Using recombinant DNA technology to engineer and produce a hemoglobin-based therapeutic (1) eliminates the risk of infectious disease transmission, (2) avoids the need for chemical modifications, (3) improves oxygen-delivery capability, and (4) allows the therapeutic compound to be manufactured in unlimited quantities with competitive economics.

Preclinical studies

Efficacy

Optro has demonstrated superior capability of delivering oxygen to tissues in two preclinical experimental paradigms: total isovolemic exchange transfusion in rodents and resuscitation from acute hemorrhage in dogs and rodents.

In the isovolemic exchange transfusion study [13], rats were subjected to continuous exchange transfusion over a period of ~40 min with either 5% (w/v) human serum albumin or 3% (w/v) Optro, both in phosphate buffered saline. Throughout the period of exchange transfusion and for an additional 4–5 h, the brain and gut of the rats were monitored non-invasively for cellular high energy phosphates (ATP and

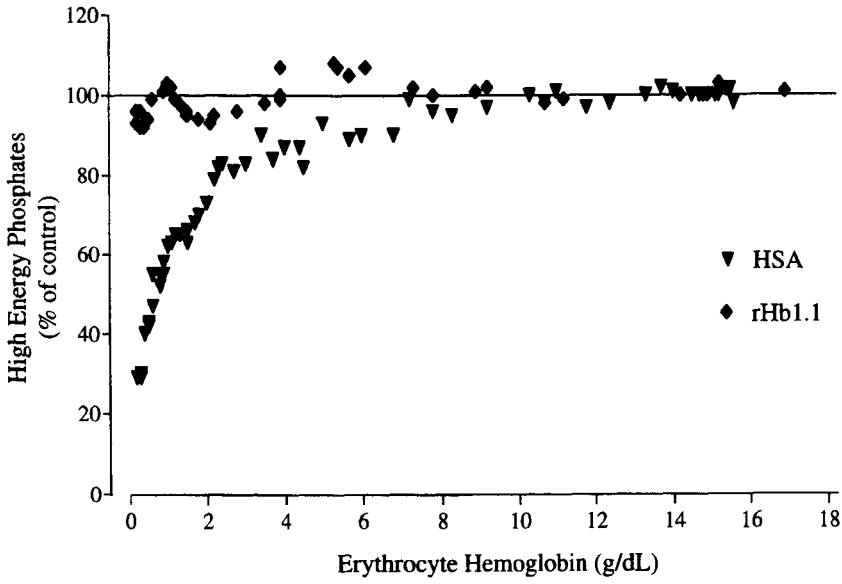

Fig. 2. Support of oxidative phosphorylation in live animals by rHb1.1 in the absence of red blood cells. The relative concentration of phosphorous present in high energy molecules (ATP and phosphocreatine) was determined by ^{31}P-NMR spectroscopy of unconscious rats as they are hemodiluted with either 5% human serum albumin or 3% rHb1.1.

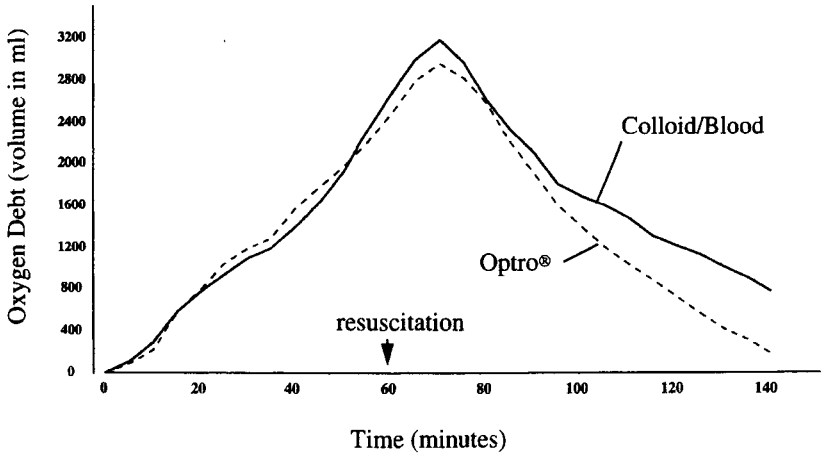

Fig. 3. Reversal of oxygen debt upon resuscitation with rHb1.1 after severe hemorrhage. Oxygen debt was continuously measured in anesthetized dogs throughout a period of severe hemorrhage (removal of 60–70% of blood volume) followed by a period of resuscitation with either 5% rHb1.1 or 5% albumin/shed blood mixture. At 140 min, Optro reversed oxygen debt by 95% whereas colloid/blood reversed oxygen debt only 73%.

phosphocreatine) and pH using ^{31}P-NMR spectroscopy (Fig. 2). Animals receiving human serum albumin experienced a precipitous fall in cellular high energy phosphate and pH when their hematocrit reached 26% ± 2%. All animals in the albumin group failed to survive beyond 43 min. Animals receiving 3% Optro, however, showed no fall in cellular high energy phosphates or pH throughout the entire exchange transfusion to a hematocrit of less than 2%. Animals receiving Optro also survived the 5-h post-transfusion observation period. The critical hematocrit of 26% observed in the albumin group has an equivalent of 8–9 g/dl of hemoglobin, which is three-fold higher than 3% Optro which supported normal oxidative metabolism.

In another measure of efficacy, Optro proved to be more effective than conventional resuscitation therapy of colloid and blood at reversing oxygen debt due to severe hemorrhage [14]. In these experiments, dogs were bled over a 60 min period until approximately 70% of their total blood volume was removed. Animals in the control group were resuscitated first with colloid (60% shed blood volume) and then with shed blood (60% shed blood volume, 118 g of hemoglobin). Animals in the study group were resuscitated with 5% (w/v) Optro solution (120% shed blood volume, 85 g rHb1.1). Oxygen debt (blood lactate and base deficit) was monitored throughout the entire hemorrhage and resuscitation period plus an additional 80 min (Fig. 3).

Both groups were followed for 7 d after hemorrhage and had normal renal and hepatic function. However, at equal resuscitation volume, Optro resuscitation produced a more rapid initial fall in oxygen debt and caused a more complete washout of metabolic acids. These results suggest that Optro is more effective than

red blood cell hemoglobin at providing oxygen to ischemic tissues resulting from hemorrhage. Similar results have been obtained in rat studies.

In a separate series of experiments, sheep were bled and transfused immediately with 45 g of rHb1.1 or provided with a similar oxygen carrying content of hemoglobin in the form of autologous blood prior to placing these animals on extracorporeal cardiopulmonary bypass [15]. This amount of rHb1.1 was used to simulate the maximum human dose in current clinical trials of 100 g. Following infusion of rHb1.1, sheep were supported by cardiopulmonary bypass for 90 min under hypothermia with aortic cross-clamp and cardioplegia. Following this 90 min, cardioplegia and cross-clamp were removed, and the animals were disconnected from the pump and allowed to maintain their circulation for an additional 60 min. A complete hemodynamic profile was monitored on both groups and with the exception of hematocrits and total hemoglobin content, these animals were indistinguishable. Specifically, there were no significant differences between the control and rHb1.1 treated groups in systemic and pulmonary vascular resistance, central venous pressure, left atrial filling pressures and in alveolar to arteriolar oxygen gradients. Oxygen consumption was also monitored and the results suggested that there were no significant differences in oxygen consumption in animals undergoing cardiopulmonary bypass that had been infused with rHb1.1 to simulate 4 unit dose following removal of 54% of the total blood volume when compared to animals that received autologous blood plus colloid to match oxygen carrying content of rHb1.1. Therefore, under physiological stress associated with cardiopulmonary bypass, oxygen consumption was maintained without significant hemodynamic changes in the rHb1.1 treated group when compared to the autologous controls.

Endotoxin/sepsis

There are a number of contradictory reports regarding the interaction of endotoxin with acellular hemoglobin and the potential complications of sepsis. To resolve these issues, rHb1.1 was tested in a pre-clinical model of lethal and sub-lethal endotoxemia and low grade-bacteremia. The effects of rHb1.1 on several biochemical parameters of complications and animal survival were measured.

In the bacteremia study, rats were inoculated peritoneally with a gel capsule containing 5000 CFU of *Escherichia coli* (strain Sm 018) combined with 35 mg of sterile rat feces, 18 h prior to exchange transfusion with either rHb1.1, human serum albumin, or autologous blood. The results of this experiments (Table 1) demonstrated that mortality between these three groups was between 10–20% and were not statistically different. Furthermore, there were no significant differences in CFU or leukocyte counts in the blood throughout a 56-h time period. In contrast, an additional group of animals that was administered a non-specific nitric oxide synthase inhibitor, L-NAME, at 25 mg/kg, exhibited 60% mortality. These data demonstrate that administration of rHb1.1 at a concentration as high as 1 g/kg, does not result in exacerbation of an occult infection when tested in a clinically relevant protocol.

Table 1

Effects of rHb1.1 and autologous blood on the survival of a rat model of occult peritoneal infection

Treatment group	Survival (%)
5% human serum albumin	13/16 (81%)
Autologous blood	8/9 (89%)
5% rHb1.1	9/11 (82%)
L-NAME (25 mg/kg)	8/20 (40%)
0.9% saline	14/20 (60%)

The differences in survival results of the HSA, autologous blood and rHb1.1 groups are not statistically different.

The models for endotoxemia are far more difficult to construct in a clinically meaningful way because of the marked difference in sensitivity of rodents and humans to endotoxin, the complex physiological response to endotoxin, and the complex and heterogeneous nature of endotoxins themselves. In these experiments, mice were injected with rHb1.1 (1 g/kg), human serum albumin (1 g/kg) or phosphate buffered saline, and then challenged with lethal (20 mg/kg), or sub-lethal (10 mg/kg or 2.5 mg/kg) doses of lipopolysaccharide (LPS). The effects of rHb1.1 on both the survival and serum levels of tumor necrosis factor and interleukin-6 were determined.

These results demonstrated that mice pretreated with rHb1.1 and challenged with 20 mg/kg LPS had 100% mortality occurring by 20 h while the same mortality in the albumin and saline group did not occur until 50 h. There were no statistically significant differences in survival among groups of animals when challenged with 2.5 mg/kg LPS. Mice challenged with 10 mg/kg LPS exhibited 100% mortality if pretreated with rHb1.1 versus 17% mortality for animals in the control groups.

TNF and IL-6 levels were no different among groups of animals challenged with 2.5 mg/kg LPS. However, at the 10 mg/kg dose of LPS, mice pretreated with rHb1.1 had a two-fold increase in IL-6 levels (225 ng/ml) when compared to animals pretreated with saline (100–125 ng/ml).

These results suggest that the effects of rHb1.1 on in vivo potentiation of endotoxin-induced lethality were modest and were apparent only at supra-clinical concentrations of endotoxin.

Rheology

The impact on blood rheology of introducing a solution of recombinant hemoglobin into the blood is an important component of its efficiency in delivering oxygen to tissues. Recombinant hemoglobin, rHb1.1, as a 5% solution has a viscosity of 0.80 mPa.s at 37°C. This compares with a higher viscosity of 0.93 mPa.s for a 5% solution of human serum albumin.

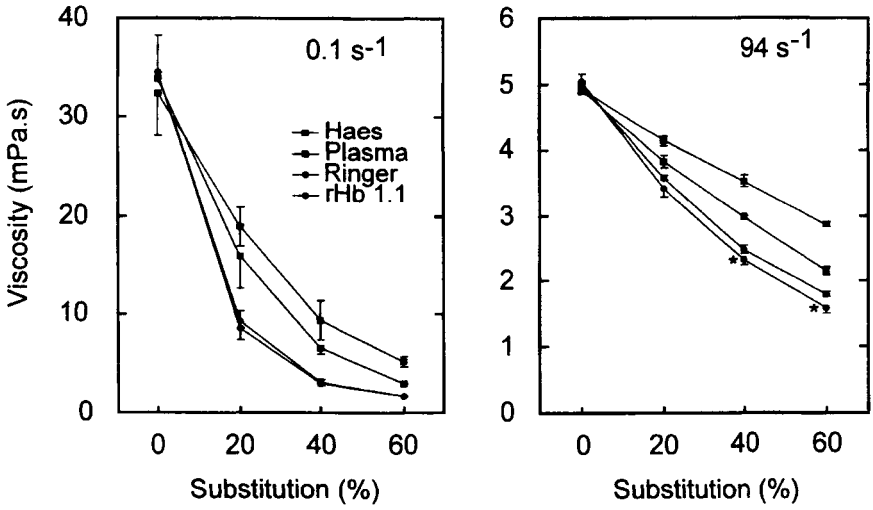

Fig. 4. Influence of rHb1.1 and volume expanders on whole blood viscosity. Whole blood was substituted with the indicated concentrations (x-axis) of Ringer's salts solution, hydroxyethyl starch, plasma or rHb1.1 and subjected to low (0.1 s^{-1}) or high (94 s^{-1}) shear rates and viscosity measured in a Couette viscometer at 37°C. While all replacing solutions decreased whole blood viscosity, the most significant reductions were observed with Ringer's and rHb1.1.

When rHb1.1 is added to human blood, there is a dose dependent decrease in solution viscosity as illustrated in the Fig. 4. The decrease in blood viscosity with rHb1.1 is greater at lower shear rates. Erythrocyte aggregation and deformability is also unchanged by the addition of rHb1.1. These results demonstrate that rHb1.1 has excellent rheological properties with whole blood, and its ability to reduce blood viscosity may provide additional benefit toward delivering oxygen to tissues, particularly in conditions of low blood flow, such as shock, trauma and ischemia.

Clinical Development

Phase I clinical trials

From 1991 through 1993, Somatogen conducted a series of trials in 103 healthy, male volunteers (86 on Optro, 17 on HSA control) to evaluate the safety and pharmacokinetics of Optro. The maximum dose reached in these safety trials was 25 g and was generally well tolerated. No evidence of nephrotoxicity, immunogenicity, coagulation changes, or significant clinical effects was observed in these studies. The plasma clearance of Optro was dose related with a half-life of 12 h at plasma concentrations of 5 mg/ml [16].

In the early stages of the Phase I studies, some volunteers given Optro reported a "flu-like" syndrome consisting of fever, chills, headache and myalgia. Symptoms typically began 4–8 h after infusion and either resolved spontaneously, or responded

to ibuprofen therapy. The "flu-like" symptoms were subsequently eliminated by a change in the manufacturing process of Optro which reduced any contaminating *E. coli* proteins and endotoxins to below detectable levels as measured by state-of-the-art analytical tools.

Systolic blood pressure increased approximately 5 to 50 mmHg in Optro dosed subjects. The blood pressure began to increase during or immediately after the infusion of rHb1.1, remained elevated until six to eight hours post infusion, and then decreased to base line levels.

Approximately half of the normal volunteers dosed with more than 5 g/kg of Optro experienced transitory mild to moderate symptoms of dysphagia, vomiting and nausea. An esophageal manometry study documented dysmotility, while other studies showed that prophylaxis with terbutaline sulfate or nifedipine was somewhat successful in reducing these gastrointestinal symptoms.

Some volunteers also experienced transitory increases in serum amylase and/or lipase, but not necessarily both. These enzymes peaked 4 h post-infusion and generally had returned to normal limits at 24 h. While rises were sometimes marked and were at times associated with mild to moderate gastrointestinal symptoms, the typical clinical picture of acute pancreatitis was not seen (severe, persistent pain and vomiting).

The transient blood pressure increases, dysphagia and amylase/lipase rises are thought to be related to nitric oxide scavenging by acellular hemoglobin. This in turn results in smooth muscle contraction which occurs in the vasculature as well as the smooth muscle of the esophagus. Transient contraction of the Sphincter of Oddi is thought to be a plausible explanation for the amylase/lipase increases, pending more definitive information related to mechanism.

Phase I/II clinical trials

Optro was given to surgical patients for the first time in 1994 in a Phase I/II trial [17]. The study was a single-center, randomized, single-blind, dose-escalating, placebo-controlled trial in elective surgery patients. Surgeries were orthopedic, maxillofacial, plastic or urological and a total of 18 patients were enrolled (14 on Optro, 4 on saline control). Dose was escalated to 25.6 g and the product was given after induction of general anesthesia when the patient was stable. Infusion was not based on physiological need.

There were no serious complications in this study and Optro was generally well tolerated. Most adverse events were similar in incidence and severity in both the Optro treated group and the saline control group and were thought to be due to the effects of anesthesia and surgery.

Transient increases in blood pressure were observed in the Optro group and resolved by 7 h post-infusion. Blood pressure rises did not correlate with dose and in those patients where therapeutic intervention was used, standard antihypertensive regimens were successful.

Five (36%) of 14 Optro and 1 (25%) of 4 saline-treated patients had elevations in serum amylase or lipase, but not both. Values generally peaked at 4–24 h and were normal at 24 h. No typical cases of acute pancreatitis were noted, though patients did report mild to moderate gastrointestinal symptoms, including nausea, vomiting, diarrhea and/or abdominal pain. (These symptoms were also common in the saline group). Of note was that the magnitude of the enzyme rises appeared lower in this study than in previous volunteer trials.

Esophageal symptoms of epigastric pain and dysphagia were also notably absent as compared to prior volunteer experience. It is likely that the anesthetic agents used provided a dilatory stimulus to smooth muscle which to some degree countered the constrictive effect caused by nitric oxide scavenging.

Early phase II clinical trials

In 1995, Somatogen conducted two Phase II trials of Optro for the following indications: intraoperative blood replacement and acute normovolemic hemodilution. These studies were focused primarily on safety. Both studies had dose-escalation designs.

Intraoperative blood replacement. This randomized, single blinded, parallel, multicenter, dose and rate escalation, controlled trial was designed to study safety and to gather preliminary efficacy/activity data in patients who experience blood loss during elective surgery [18].

A total of 38 patients (25 Optro, 13 control) were enrolled and randomized. Nine Optro and 6 control patients did not require an intraoperative transfusion and were discontinued from study participation at the end of surgery; thus, a total of 16 Optro and 7 control patients were dosed. Optro doses ranged up to 100 g and allogeneic blood was used as the control. Six Optro patients received doses of 25 g, four received doses of 50 g, three received doses of 75 g, and three received doses of 100 g. The first three patients at the 25 g dose level were infused at a controlled rate, and all other patients were infused at a rate comparable to the clinically desired rate of infusion in blood transfusions. The majority of the surgical procedures were orthopedic or urologic in nature.

There were no clinically significant, treatment-related adverse events in this clinical trial and no evidence of nephrotoxity, immunogenicity, or coagulation changes. Patients received no prophylactic pre-treatment with any drugs to manage or avoid potential symptoms or side effects. None of the patients in this study experienced the esophageal symptoms that had been seen in certain subjects in prior Phase I clinical trials. In certain patients, there were transitory elevations of amylase and/or lipase which spontaneously resolved without treatment and which investigators determined not to be clinically significant. These rises tended to peak around 12–24 h and return to baseline within 48 h. No cases of typical acute pancreatitis were seen. Transient blood pressure rises, which resolved within 12 h or less, were seen in approximately

two-thirds of Optro patients. Most adverse events were similar in incidence and severity in both the Optro treated group and allogeneic blood control group and were thought to be due to the effects of anesthesia and surgery.

Acute normovolemic hemodilution. This single-center, single blinded, randomized, placebo-controlled, parallel, dose-escalation study was designed to measure safety and to gather preliminary efficacy/activity data in patients who donate blood via an acute normovolemic hemodilution procedure just prior to surgery [19].

A total of 11 patients were enrolled, however 1 patient was discontinued prior to treatment administration due to the unavailability of study drug. Thus, 10 patients received study drug or comparator (7 Optro, 3 control). Three patients received 12.5 g, one patient received 25 g and three patients received 50 g of Optro. Saline was used as the control.

Patients in this trial received no prophylactic pre-treatment with any drugs to manage or avoid potential symptoms or side effects of Optro. There were no clinically significant, treatment-related adverse events, and no esophageal symptoms in any of the patients in this study. As in the intraoperative blood replacement trial, a few (2) patients experienced transitory elevations of amylase and lipase levels, which spontaneously resolved without treatment, had a similar time course to other patient studies, and which investigators determined not to be clinically significant. Classical acute pancreatitis was not seen. Most adverse events were similar in incidence and severity in both the Optro treated group and saline control group and were thought to be due to the effects of anesthesia and surgery.

Late Phase II clinical trials

In April 1997, Somatogen commenced patient enrollment in a multi-center, randomized, double-blind, parallel, dose-escalation Phase II study of Optro to explore the safety and preliminary efficacy of enhanced ANH in cardiac surgery. These studies will, when completed, examine the safety and efficacy of removing up to four units of autologous blood via acute normovolemic hemodilution, with Optro replacing the volume withdrawn. Approximately 50 patients undergoing coronary artery bypass graft surgery will be enrolled in these studies at five to eight sites throughout the US.

Enhanced ANH in cardiac surgery. Each year in the US, approximately 500,000 patients undergo cardiac surgery for coronary artery bypass grafting, atrial septal defect repair, or valve replacement. These procedures are highly traumatic to the patient both in terms of the surgical insult itself as well as an "inflammatory" effect created by exposing the patient's blood to the CPB machine, which results in significant post-operative bleeding and other complications [20,21]. A procedure called acute normovolemic hemodilution (ANH) is frequently used in cardiac surgery to sequester the patient's blood before going on bypass to minimize exposure to the CPB machine [22]. Somatogen believes that the use of Optro in this setting may allow

greater amounts of the patient's blood to be harvested, thus decreasing post-operative blood use and bleeding complications.

Scale-up and manufacturing

Recombinant hemoglobin rHb1.1 is produced by fermentation of a strain of *E. coli* derived from JM107, transformed with a plasmid containing a synthetic operon composed of the di-alpha-globin and beta^{Pres}-globin genes transcribed from the tac promoter [23]. The plasmid contains a tetracycline resistant gene with pUC high copy number origin of replication [24].

More recently, in preparation for final commercial manufacturing, the *E. coli* production strain has been further modified for phage resistance and the addition of an extra copy of the gene for ferrochelatase (hemH) under the control of the *tac* promoter [25]. The extra hemH gene allows for the increased production of the enzyme that converts protoporphyrin IX to heme.

Fermentation is performed in a defined medium in a 1500 liter fermentation vessel generally as described by Looker et al. [11]. Induction of expression is achieved by addition of IPTG (isopropyl thiol galactopyranoside) in log growth phase and hemin is added to achieve maximal expression levels.

rHb1.1 is produced and accumulated in the *E. coli* as a soluble, functional, assembled pseudotetramer (di-alpha globin, two beta-globins and four heme groups). At the end of the induction period, approximately 15% of the *E. coli* cell protein is rHb1.1 [26]. These expression results are quite unusual since fermentation of recombinant proteins in *E. coli* generally results in the accumulation of the foreign protein as an insoluble, inactive product which must be carefully refolded after purification. These results are even more surprising given the complexity of hemoglobin.

rHb1.1 is purified from the fermentation mixture in a straightforward process which employs a minimum of steps, all of which are high yielding and easily scaleable. The *E. coli* are fractured to liberate the rHb1.1 into the milieu by flowing the contents of the fermentor through a continuous mechanical homogenizer. The fluid stream is also passed through a heating coil to kill remaining viable *E. coli* and to precipitate unwanted proteins which have less thermal stability than rHb1.1. The debris from the *E. coli* fracturing and heat precipitation are removed by rotary filtration or continuous centrifugation. At this stage, the effluent stream is a red solution containing soluble rHb1.1 at a purity of approximately 20%.

The final purification steps employ two large-scale chromatography columns: first a zinc-ion-chelate affinity capture resin followed by an anion exchange resin. These final two steps remove all traces of *E. coli* proteins and other contaminants. The purified rHb1.1 is diafiltered into phosphate buffered saline and adjusted to a concentration of 5% protein (w/v). The final product is >99.99% pure with a p_{50} of 31–33 mmHg, n_{max} of 2.3–2.4, and less than 3% met-hemoglobin (Fig. 5).

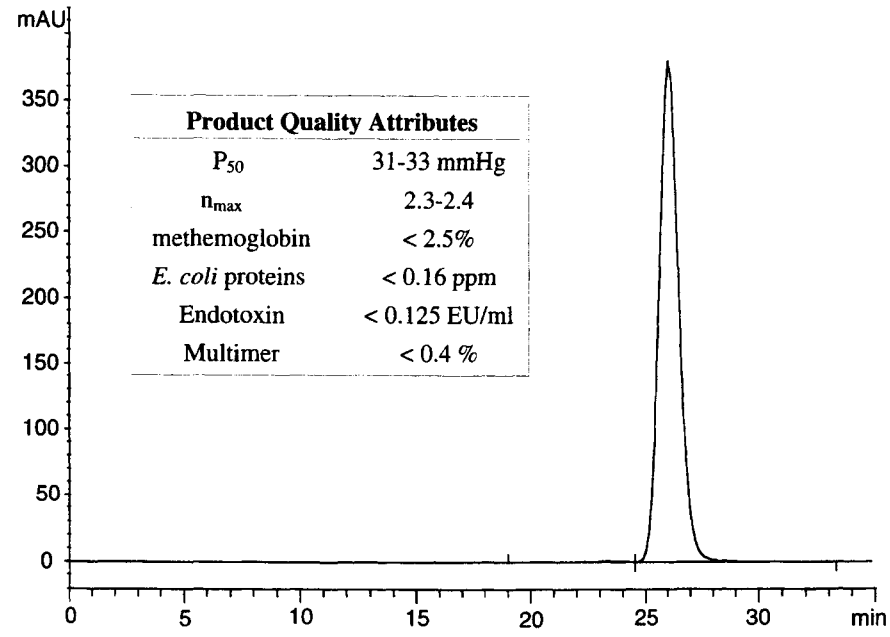

Fig. 5. Homogeneity and purity of rHb1.1. Size exclusion chromatogram of clinical grade rHb1.1 on high pressure Superose.

This recombinant manufacturing approach offers a number of advantages in the production of a pharmaceutical product:

1. The raw materials are well characterized and they are available in virtually unlimited quantities. The raw materials are predominan components of the fermentation media, namely, sugar and common chemicals.
2. The process can be scaled to virtually any size. The current fermentation of rHb1.1 has been scaled from 1500l liters to 50,000l with comparable results. Additional capacity can be achieved by adding additional vessels. All of the downstream purification processes are either continuous or easily scaleable to almost any dimension.
3. The process produces high quality and consistent results (Fig. 6). The quality control chart shown in the insert (Fig. 5) is an example of the purity of 20 consecutive manufacturing runs performed at 1500l scale. Both *E. coli* proteins and endotoxin levels routinely test below the limit of detection of the assays.

Extensive analytical characterization of the protein also demonstrated a high fidelity translation of rHb1.1. Because of these manufacturing characteristics, rHb1.1 meets the guidelines of a "Specific Biologic" as defined by the FDA and therefore avoids the challenges facing biologically-derived therapeutics (blood products).

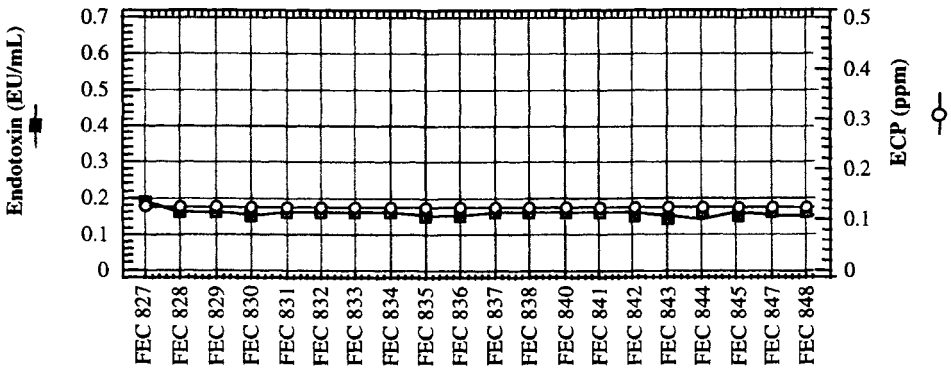

Fig. 6. Reproducibility and consistency of production of rHb1.1. Control charts monitoring the purity (endotoxin and *E. coli* proteins levels) of twenty consecutive production campaigns of rHb1.1 at 1500 liter fermentation. The lower limit of quantification of ECP is 0.16 ppm and 0.125 EU/ml for endotoxins.

Next generation recombinant hemoglobins

As demonstrated with rHb1.1, genetic engineering and recombinant expression allows protein molecules to be carefully altered to impart physiochemical and pharmacologic properties that are optimal for therapeutic use. With rHb1.1, the alpha globin chains were fused genetically with a glycine bridge to stabilize the tetramer and a single amino acid in the beta globin chains was changed to confer the appropriate oxygen affinity.

Molecular engineering can be applied to further optimize oxygen delivery, modify nitric oxide binding, increase vascular retention, optimize oncotic properties, enhance molecular stability and increase circulating half-life. Somatogen has focused its efforts over the past 12–18 months to develop a recombinant hemoglobin molecule which carries oxygen efficiently but exhibits reduced nitric oxide scavenging pharmacology.

Nitric oxide acts as a chemical messenger in the control of many important physiologic processes, including neurotransmission, inflammation, platelet aggregation, and regulation of gastrointestinal and vascular smooth muscle tone. The biological actions of nitric oxide are mediated by binding to and activation of soluble guanyl cyclase, which initiates a biochemical cascade resulting in a variety of tissue-specific responses [27]. In gastrointestinal and vascular smooth muscle, the increase in cyclic GMP concentration causes a decrease in intracellular calcium concentration, which relaxes the muscle.

Mild hypertension and gastrointestinal dysmotility has often been observed following administration of acellular hemoglobin [16,28]. Many investigators have concluded that this pharmacologic property of acellular hemoglobins is due to depletion of nitric oxide in the wall of the vasculature and in the neuromuscular

space in the gastrointestinal tract. As such, this hypothesis requires hemoglobin to extravasate into endothelial cells or interstitial spaces.

The approach taken by Somatogen has been two fold:

1. increase the molecular size of hemoglobin to restrict its extravasation and access to the extravascular compartment where nitric oxide functions, and
2. reduce the interaction of nitric oxide with the heme moieties of hemoglobin by modifying the heme pocket through selective mutation of amino acids surrounding the pocket.

Size

Genetic engineering of recombinant hemoglobin provides a myriad of approaches for creating larger molecular constructs of hemoglobin which are homogeneous and well characterized. This is contrasted with random crosslinking chemistry which produces a heterogeneous mixture of polymers with different size and function.

One approach is to replicate the strategy used with stabilizing rHb1.1 wherein the alpha subunits are fused together genetically. Fusing two di-alpha constructs will produce a "di-hemoglobin" (128 kD), fusing three di-alpha constructs will produce a "tri-hemoglobin" (192 kD), and fusing four di-alpha constructs will produce a "tetra-hemoglobin" (256 kD). All three of these macromolecular constructs have been successfully prepared, but only the di-hemoglobin has been characterized at this point [29].

Di-hemoglobin. The di-hemoglobin molecule is comprised of a self-assembled oligomer of one tetra-alpha globin and four beta globin chains. The alpha globin domains of the single polypeptide are linked as follows:

$$\propto \text{I-Gly-} \propto \text{II-SerGlyGlySerGlyGlySer-} \propto \text{III-Gly-} \propto \text{IV}$$

Di-hemoglobin was expressed at high levels in *E. coli* and readily purified from bacterial lysate to greater than 95% homogeneity. The di-hemoglobin had a molecular weight of 130 kD as determined by sedimentation equilibrium and possessed eight heme moieties as determined by carbon monoxide titration. The p_{50} and n_{max} were 24 mmHg and 2.0, respectively.

Pharmacokinetic measurements of di-hemoglobin in rats demonstrated an increase in circulating half-life of 1.4 fold over that observed for monomeric hemoglobin. Peritoneal lavage after di-hemoglobin administration in mice showed that approximately 30% less di-hemoglobin had entered the peritoneal cavity as compared to results for monomeric hemoglobin. The hemodynamic response of di-hemoglobin when administered to rats at a dose of 350 mg/kg demonstrated an increase in mean arterial pressure of $\sim$20 mmHg as compared to $\sim$30 mmHg for monomeric hemoglobin.

These results with di-hemoglobin suggest that increasing the molecular size of hemoglobin from 64 kD to 128 kD, the molecule exhibits substantially reduced

extravasation, resulting in a ~30% reduction in vasoactivity, presumably through reduced nitric oxide scavenging.

Tetra-hemoglobin. Another strategy for producing larger molecular constructs of hemoglobin employs site-specific crosslinking. Because the alpha domains of hemoglobin can be constructed and expressed as a single polypeptide chain, then a single amino acid substitution can be placed in one location for site-specific chemical crosslinking. In this model, a single cysteine residue was coded into a di-di-alpha construct (described above for di-hemoglobin) at a position known to impart high chemical reactivity. Di-hemoglobin with this cysteine mutation was expressed and purified to homogeneity. Tetra-hemoglobin was then produced by chemically crosslinking two di-hemoglobins through the reactive surface cysteine residue using bismaleimidohexane, a 16 Å sulfhydryl-specific crosslinker [30].

The tetra-hemoglobin was purified to homogeneity and tested in rats for hemodynamic pharmacology and circulating half-life. The observed t1/2 was 4.5 h versus 2.9 h for monomeric hemoglobin. The increase of mean arterial pressure observed for tetra-hemoglobin was ~15 mmHg, approximately half of that observed for monomeric hemoglobin.

These results were somewhat surprising given the fact that tetra-hemoglobin is four times the size of mono-hemoglobin (256 kD versus 64 kD). With this large difference in size, one might expect a greater increase in half-life and a greater reduction in vasoactivity if extravasation is an important component of the pharmacokinetics and smooth muscle pharmacology of acellular hemoglobins. Further work in this approach is underway to better define and quantify the relationship of extravasation with circulating half-life and nitric oxide scavenging.

Heme pocket variants

Work by scientists in the laboratories of Somatogen and Dr. John Olson at Rice University have suggested that the oxidative reaction of nitric oxide with the bound oxygen of oxyhemoglobin may be of greater significance in nitric oxide scavenging than the simple binding of nitric oxide to the iron atom [31]. In this reaction, the nitric oxide molecule does not bind to the heme, but reacts with the bound oxygen of the oxyhemoglobin complex to form methemoglobin and nitrate. The chemistry is analogous to the rapid reaction of nitric oxide with free superoxide in solution. Both the heme iron and the nitric oxide become oxidized by the bound oxygen atoms, and the reaction occurs so rapidly that no replacement of oxygen by nitric oxide is observed [31].

The reactivity of hemoglobin toward nitric oxide can be altered by making amino acid substitutions in the distal heme pocket of both subunits. When larger, more bulky amino acids are inserted into the distal heme pocket, the reactivity of the resultant hemoglobin (in a rHb1.1 framework) to nitric oxide was profoundly reduced. By repeating this process for a large number of mutant constructs, mutant subunits can be paired to achieve a wide range of rate constants ($k'_{NO,ox}$) for nitric

oxide reactivity. Substituted alpha and beta subunits with approximately equal values of $k'_{NO,ox}$ were combined into tetrameric hemoglobin constructs for testing in animals.

The reactivity to nitric oxide of three of these heme pocket mutants is illustrated in Fig. 7(a) below. The values of $k'_{NO,ox}$ for these three mutants are 24, 15 and 2 $\mu M^{-1} s^{-1}$ compared to 60 $\mu M^{-1} s^{-1}$ for several hemoglobin variants (rHb1.1, rHb0.1, rHb 0.0, and rHb Bethesda) with normal heme pockets, but very different p_{50} (32, 10, 15 and 3 mmHg, respectively). All three of these heme-pocket mutants have oxygen dissociation curves that are significantly left-shifted compared to rHb1.1. The low p_{50}s simplify the interpretation of blood-pressure experiments by minimizing the formation of deoxyhemoglobin.

The mean arterial pressure responses elicited by rHb1.1 and each of the three heme-pocket mutants are shown in Fig. 7(b). Clearly, the magnitude of the pressor response decreased as the rate constant for nitric oxide scavenging was decreased. At the lowest rate of nitric oxide oxidation, the pressor response was nearly as low as that observed following administration of an equivalent volume of 5% human serum albumin. These effects were not due simply to the low p_{50} values for the heme pocket mutants since the pressor effect of rHb1.1 (p_{50} = 32 mmHg) was identical to rHb Bethesda which has a p_{50} of 3 mmHg.

In addition, these results indicate that oxygen delivery to arteriolar smooth muscle or parenchymal tissue cannot be the mechanism of the observed pressor effect [32].

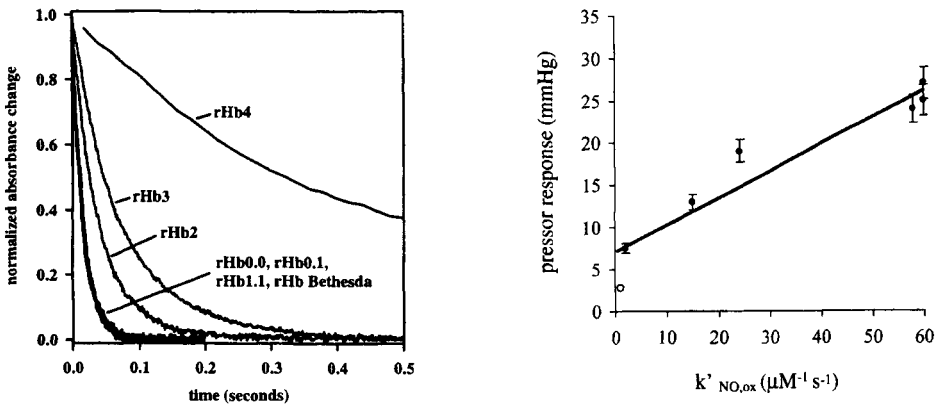

Fig. 7. (a) Normalized time courses of nitric oxide oxidation of recombinant hemoglobins. rHb0.0 is wild type human hemoglobin prepared by *E. coli* fermentation. rHb0.1 contains the di-alpha crosslink present in rHb1.1, but not the Presbyterian mutation at position β-108. rHb1.1, rHb0.1, rHb0.0 and rHb Bethesda all have wild-type amino acids in their heme pockets and all have a value for $k'_{NO,ox}$ of ~ 60 $\mu M^{-1} s^{-1}$. The distal heme pocket mutants rHb2, rHb3, rHb4 have values for $k'_{NO,ox}$ of 24, 15, and 2 $\mu M^{-1} s^{-1}$, respectively; (b) Pressor effects of recombinant hemoglobins with heme pocket mutations and human serum albumin. Mean arterial pressure was continuously monitored in rats after administration of 350 g/kg of various heme pocket rHbs or human serum albumin. The various rHbs are identified with their respective $k'_{NO,ox}$ values. There is a linear correlation between $k'_{NO,ox}$ and pressor effect in rats.

The p_{50} of rHb Bethesda is low enough that this hemoglobin is essentially incapable of delivering oxygen to tissues, especially in top-load experiments where a normal complement of erythrocyte hemoglobin is present and maintains normal tissue pO_2 levels. These results indicate that the mechanism of the pressor response of acellular hemoglobins is depletion of nitric oxide, not excessive oxygen delivery to arterioles.

Having demonstrated the importance of nitric oxide scavenging, we took the final step to engineer recombinant hemoglobins that deliver oxygen efficiently while having significant reduced rates of reaction with nitric oxide. We have discovered that placing bulky amino acids in the distal heme pocket to slow down nitric oxide reactivity does not consistently slow down the dissociation of oxygen from oxyhemoglobin. We also focused on modifying the allosteric equilibrium of hemoglobin with mutations at key positions away from the heme pocket to effect efficient oxygen off-loading. Such changes in hemoglobin allostery are specific to oxygen kinetics and affinity, since the "R/T" equilibrium has no effect on nitric oxide scavenging.

Several double mutants which have a modified heme pocket and an altered R/T equilibrium have now been prepared and are being tested. Preliminary results show a substantially reduced vasopressor effect and efficient oxygen delivery. These hemoglobin variants are being scaled up for more thorough characterization and preclinical testing. In a parallel effort, these nitric oxide variants are also being engineered and expressed as di-hemoglobin constructs to further gain the benefit of increased size and reduced extravasation.

Summary

Applying genetic engineering and recombinant expression to the development of hemoglobin-based oxygen therapeutics offers tremendous flexibility in optimizing physiochemical and pharmacological properties of the drug. The first example of this is rHb1.1 which is a molecular variant of human hemoglobin. This compound is being tested in Phase II clinical trials as an oxygen therapeutic for enhancing acute normovolemic hemodilution in cardiac surgery. rHb1.1 has proven to be more efficient than whole blood in supporting oxidative phosphorylation and reversal of oxygen debt in animals models of extreme anemia and hemorrhagic shock, respectively. Human patients have been dosed with up to 100 g of rHb1.1 with no untoward clinical complications attributable to the product.

Next generation recombinant hemoglobin compounds with modified nitric oxide scavenging have now been produced and are in the early stages of testing and development as therapeutic agents for preventing and treating tissue hypoxia resulting from acute blood loss and vascular ischemia.

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Blood Substitutes — Present and Future Perspectives
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CHAPTER 6

Overview of the Effects of Diaspirin Crosslinked Hemoglobin (DCLHb) on Oxygenation, Perfusion of the Microcirculation, and Clinical Studies

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Introduction

The purpose of this overview is to discuss some of the aspects of the preclinical and clinical development of diaspirin crosslinked hemoglobin (DCLHb). DCLHb belongs to a new class of agents, referred to by many regulatory agencies as hemoglobin-based oxygen carriers (HBOCs). These solutions were originally developed as "blood substitutes", a term commonly used in the lay press. While this term has a certain simplistic appeal, data accumulated from a number of different laboratories demonstrate that DCLHb might more accurately be described as a "hemoglobin therapeutic". There is a considerable difference in these two concepts.

Solutions such as DCLHb are not complete substitutes for blood because they do not have clotting factors, protease inhibitors, antibodies, other proteins routinely found in blood, such as complement and other transport proteins, or blood-formed elements. However, they do avoid some of the potential problems that are commonly associated with red blood cell transfusion. For example, blood requires extensive testing before use, while solutions such as DCLHb do not require type- and cross-matching prior to infusion.

Although the safety of the blood supply has improved dramatically, there are still concerns regarding the transmission of blood-borne disease. In fact, concern regarding transmission of viral diseases such as HIV was one of the primary driving forces behind the research and development of these solutions. To address the issue of potential viral contamination, the production of DCLHb utilizes extensively tested human blood that has been deemed acceptable for transfusion. In addition, two robust viral-reduction steps (heat pasteurization and nano-filtration) are included in the manufacture of DCLHb.

A further problematic issue associated with blood is that it has a relatively short shelf-life. At present, most blood can be stored for only 42 d. Hemoglobin therapeutics, such as DCLHb, may be stored for at least one year. Perhaps of even greater

importance is the fall in the levels of 2,3-diphosphoglycerate (2,3-DPG) in the red cell during storage of blood. In fresh blood, 2,3-DPG helps maintain the hemoglobin tetramer in the proper low affinity conformation to allow easy off-loading of oxygen. However, upon transfusion, blood stored for two weeks or longer exhibits impaired oxygen transport compared with fresh blood secondary to the loss of 2,3-DPG [1]. In contrast, even when infused one year after production, DCLHb retains the ability to deliver oxygen effectively to tissues [2].

Finally, it is known that infusion of stored blood may cause immune suppression and that stored red cells may lose their ability to deform, which can also affect perfusion of small vessels. These adverse effects are not seen following infusion of DCLHb [3].

Combined with consistent and effective oxygen-carrying properties, DCLHb also has plasma volume-expansion capabilities. DCLHb is prepared as a 10 g/dl solution, which is hyperoncotic, having an oncotic pressure of approximately 42 mmHg versus a normal oncotic pressure of 27 mmHg for blood. Therefore, following infusion, DCLHb will tend to draw fluid into the vascular space. Furthermore, the product has additional pharmacological properties, such as a pressor effect on blood vessels, which supports the use of the descriptor "hemoglobin therapeutic" rather than "blood substitute". As such, DCLHb has the potential to address medical needs that are unmet by current intravenous therapy with solutions such as lactated Ringer's, saline, or blood.

Properties of DCLHb

DCLHb, ultimately to be marketed under the name HemAssist^{TM}, does not require type- or crossmatching prior to infusion since the membranes of the donated red cells have been removed. DCLHb is subjected to rigorous viral inactivation steps and has a molecular weight of approximately 64,500 D. DCLHb is stable for more than a year in the frozen state [2], and has a circulating half-life of less than one day in humans.

Manufacture

To intramolecularly crosslink the hemoglobin molecule and produce DCLHb, Baxter Healthcare Corporation completes the reaction in the presence of a polyanion and uses an activated 3,5-dibromosalicylate (DBS) diester of fumaric acid (DBBF). This agent specifically crosslinks the hemoglobin at the lysine 99 position of the two α chains (Fig. 1). A simple four carbon fumarate bridge is introduced between the two α chains. The DBS is released during hemoglobin crosslinking and is washed away during the manufacturing process subsequent to crosslinking. Consequently no aspirin is present in the final product. The result of this technique is a very simple and specific chemical modification.

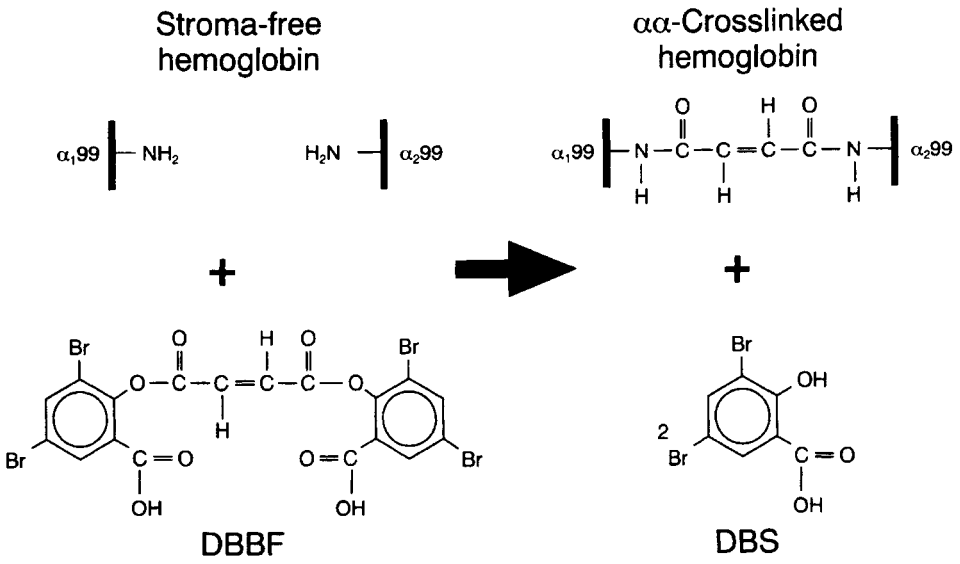

Fig. 1. Illustration of the specific chemistry adopted by Baxter Healthcare Corporation to produce diaspirin crosslinked hemoglobin (DCLHb) (also referred to as HemAssist™). Under the specific conditions established during the reaction, the DBBF (3,4-dibromosalicyl-bis-fumarate) reagent specifically reacts at the lysine 99 positions of the two α chains of stroma-free hemoglobin, leading to the production of α-α crosslinked hemoglobin (i.e. crosslinked by a simple four carbon fumarate bridge). The DBS (dibromosalycilate) is a process residual that is washed away following the crosslinking reaction.

The fumarate crosslinking serves several purposes: firstly, by crosslinking the hemoglobin tetramer, the oxygen-binding affinity is reduced to physiologic levels, and the oxygen-dissociation curve is right-shifted [4–6]. The p_{50} of the DCLHb is approximately 32 mmHg, compared with approximately 28 mmHg in normal blood. Therefore, it is easier for DCLHb to offload oxygen than it is for stored or fresh human blood. Secondly, the crosslinking reaction also stabilizes the hemoglobin molecule so that the crosslinked product can be subjected to temperatures even higher than those routinely used in the pasteurization of human serum albumin. This heat treatment step achieves two significant goals: (1) precipitation of proteins in blood, leading to a very highly crosslinked and pure final product [7], and (2) very effective viral inactivation. Utilizing a broad spectrum of models and relevant enveloped and non-enveloped viruses, both the heat treatment step and an ultrafiltration step used in the production process have been validated regarding virus removal or inactivation, with combined virus titer log reduction values of at least 10^{12} [8].

It is also notable that the fumarate α–α crosslink is located in the center of the hemoglobin molecule (Fig. 2), a position which is highly unlikely to make DCLHb antigenic or immunogenic.

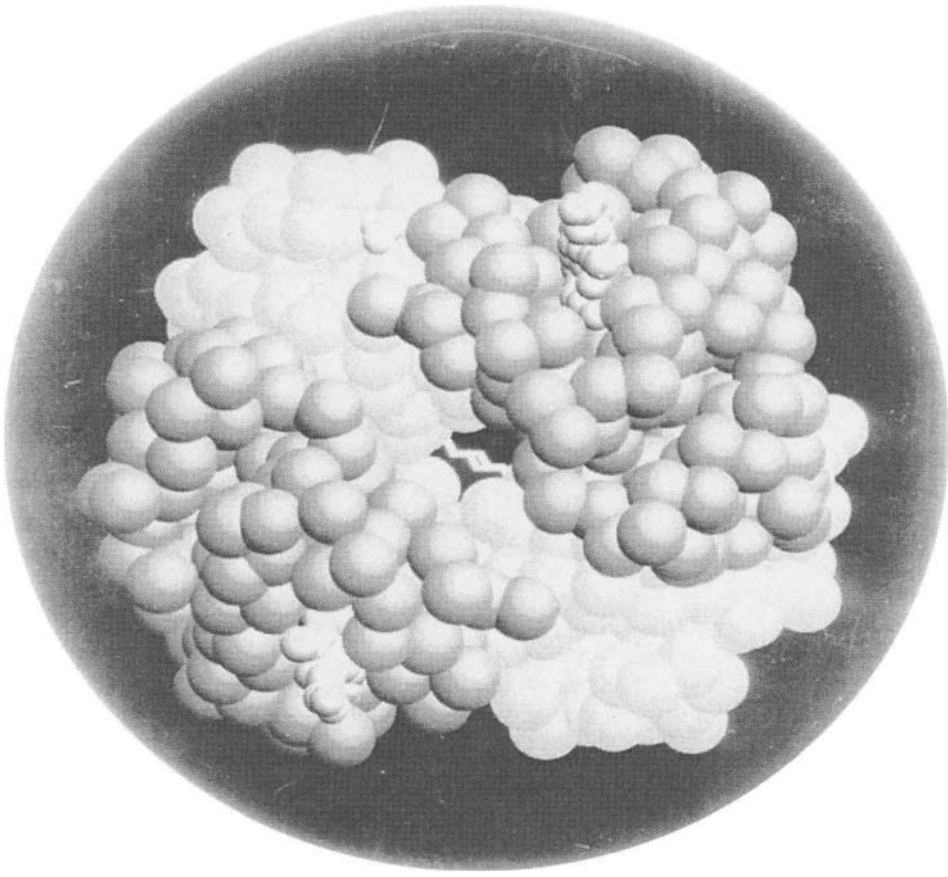

Fig. 2. Model illustrating the molecular structure of the Baxter Healthcare Corporation hemoglobin therapeutic, DCLHb. The molecule consists of two alpha (light blue) and two beta (dark blue) subunits and four heme groups (red). The heme groups each contain an iron atom where oxygen binds to the molecule. The patented crosslink (center) stabilizes the molecule, enabling it to deliver oxygen to tissues in a manner similar to whole blood.

Physical properties

Half-life. The half-life of DCLHb is 12–24 h at clinically useful doses in humans, considerably less than that of red blood cells. However, despite the short half-life of these products, their biological activity is more than sufficient for these products to serve as a bridge to transfusion in critically ill patients.

Interference with laboratory assays. DCLHb is a red, translucent solution, similar in appearance to blood. Unfortunately, the red color means that it is extremely difficult to conduct a blinded trial with DCLHb. Even if the bag and infusion lines are

covered, as soon as a patient or animal is infused with the product, the hemoglobin enters the plasma component of the whole blood. In addition, the red color in the sample interferes with many routine laboratory assays, since many of these assays are spectrophotometric. The hemoglobin in DCLHb absorbs close to the same wavelength as many of the different analytes needed to be measured. These issues are not insurmountable but do create difficulties in certain cases.

Pharmacological properties

Another important property of DCLHb, and one of the primary reasons it should be classified as a hemoglobin therapeutic, is its pharmacological activity following infusion (Fig. 3). In several different animal species, DCLHb causes a rapid increase in mean arterial blood pressure (MAP). This pressor response occurs at very low volumes and is rapid in onset, but unlike most pressor agents, is self-limiting in

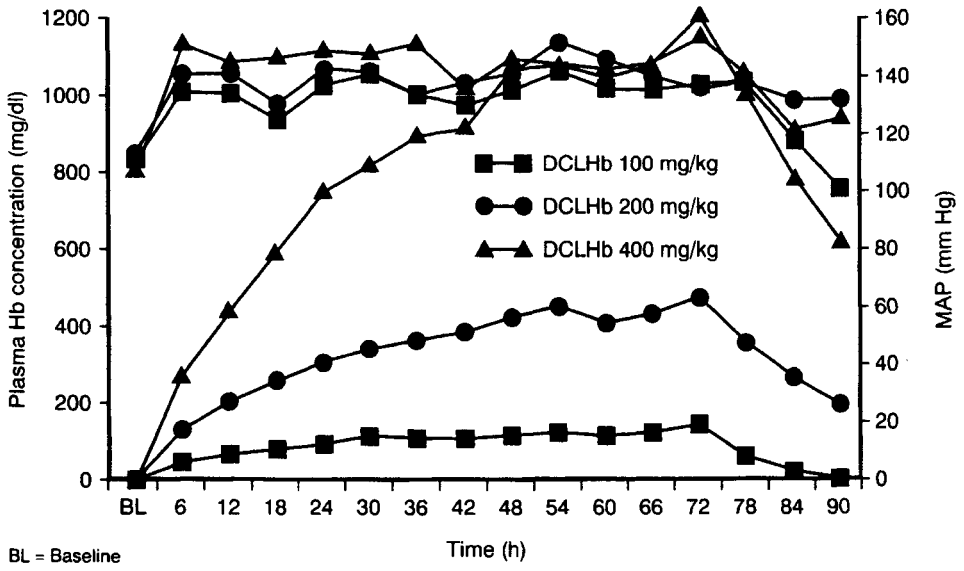

Fig. 3. Effects of repeated doses (i.e. 100, 200 or 400 mg/kg) of DCLHb infused intravenously at a rate of 1 ml/kg/min every 6 h for 66 hours to awake domestic swine (four pigs/group). After the last dose of DCLHb, the animals were monitored for an additional 24 h. Shown are the changes in the measured plasma hemoglobin concentrations (mg/dl) and mean arterial blood pressure (MAP; mmHg) over time (in hours). As can be seen, following the initial infusion of the three different doses of DCLHb, MAP increased to a similar amount in all three groups, despite the incremental increases in plasma hemoglobin concentrations. Likewise, following subsequent infusions of different doses of DCLHb, despite the dramatic dose-dependent increase in plasma hemoglobin concentrations, the MAP quickly reached a "plateau", and did not increase further with increasing doses, clearly illustrating the self-limiting nature of the DCLHb-induced pressor response. As the DCLHb was cleared from the circulation (i.e., after the last dose), MAP slowly began to return towards baseline levels.

nature: after relatively low doses, further administration of the product does not result in additional increases in blood pressure.

DCLHb: Preclinical summary

A number of techniques have been employed to assess the effects of DCLHb on the perfusion of the microcirculation. These include hemodynamic measurements, acid-base status, radioactive microsphere measurements, blood flow measurement with ultrasonic flow probes, and intravital microscopy. Oxygenation of key tissues may be assessed via palladium porphyrin phosphorescence techniques, micro-platinum electrodes placed in the muscle tissue, oxygen-sensing electrodes (optodes), and measurement of whole body oxygen consumption.

In a study conducted by Dr Gulati from the University of Illinois at Chicago, blood flow in normovolemic animals was studied using radioactive microspheres [9]. After DCLHb infusion, there was a slight decrease in blood flow to the skin and muscle. However, there was an increase in blood flow to most of the vital organs, particularly the heart and gut. An increase in blood flow to these key shock organs, with a slight diversion of blood flow from muscle, is likely to be beneficial, particularly in trauma/hemorrhagic shock patients. Furthermore, because approximately 40–50% of the cardiac output goes to the muscle, small changes in the amount of blood flowing to this tissue could cause a significant redistribution of blood flow to other key organs. Skeletal muscle has a large mass and already has luxury flow above and beyond basic metabolic demands, whereas organs such as the heart, have a very small mass but have a large need with very little luxury blood flow.

DCLHb in animal models of hemorrhagic shock

Studies of DCLHb in animals with hemorrhagic shock have demonstrated the ability of this compound to quickly restore mean arterial blood pressure, base deficit [10], and subcutaneous and mucosal pO_2 to baseline levels. In addition, DCLHb helps preserve normal gut architecture, decreases bacterial translocation, increases blood flow to key tissues and organs [9,11], and decreases mortality in these models [12].

As noted above for normovolemic animals, administration of DCLHb to hemorrhaged animals may serve to create a small shift in blood flow/perfusion from the muscle, resulting in a large increase in flow to vital organs. This shift may have a significant beneficial effect in certain indications, such as shock, and result in maintenance of vital organ perfusion [12]. A variety of techniques have been used to evaluate perfusion following infusion of DCLHb; the common conclusion from all of the studies is that vital organ perfusion consistently is maintained. This property of DCLHb may be explained in part by examining its pharmacological characteristics; DCLHb is not simply an inert protein that carries oxygen. The pressor action of hemoglobin involves a number of different autocrine systems in the body, including effects/interactions with nitric oxide, endothelin, and the α-adrenergic system (Fig. 4).

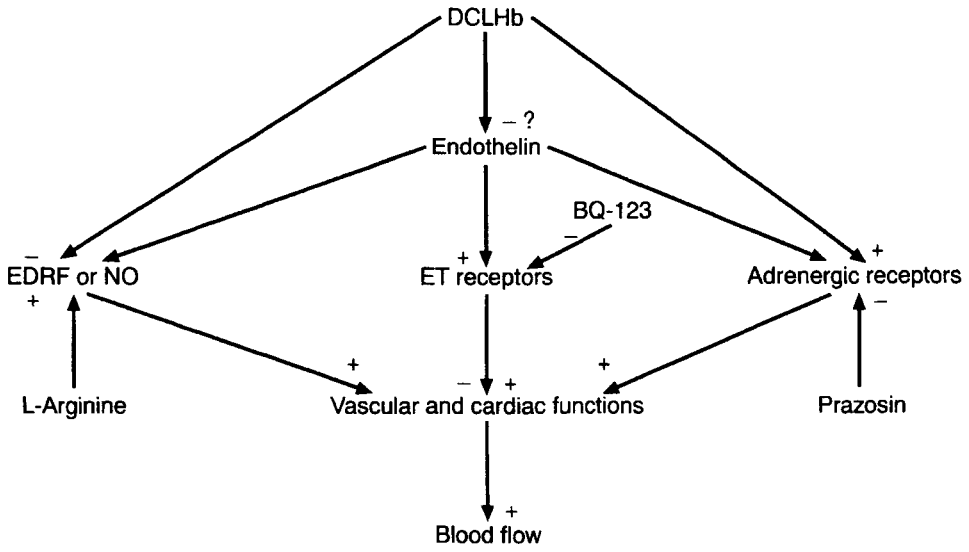

Fig. 4. Schematic representation of the proposed mechanism of action of the cardiovascular effects of DCLHb. NO = Nitric oxide or endothelium derived relaxing factor (EDRF); ET = Endothelin; BQ-123 = Specific ETA receptor antagonist. The scheme illustrates the potential complex interaction of a number of endogenous, natural autocrine pathways that appear to be involved in the cardiovascular responses of DCLHb.

DCLHb appears to work through multiple endogenous systems and mechanisms, each of which probably interact.

A further study of DCLHb as a low-volume resuscitation agent in severe hemorrhagic shock in pigs demonstrated the agent's ability to improve gut microvascular oxygenation [13]. In this study, Pd porphyrin phosphorescence was used as a marker of microvascular pO_2. DCLHb restored microvascular oxygenation to pre-hemorrhagic levels.

Effects of DCLHb on the microcirculation of striated skin muscle

The effects of DCLHb have been investigated on microvascular alterations and local tissue pO_2 in striated skin muscle of the hamster after severe ischemia followed by reperfusion [14]. Intravital fluorescence microscopy and a multi-wire surface oxygen electrode (MDO, Eschweiler, Kiel, Germany) were used in the striated skin muscle preparation of the awake Syrian golden hamster for quantitative analysis of microcirculatory parameters (vessel diameter, venular leukocyte/endothelium interaction, macromolecular leakage, functional capillary density) and local tissue oxygen tension (tpO_2), respectively. Animals ($n = 8$ per group) were subjected to a 4-h local pressure ischemia followed by 24 h of reperfusion. Animals were treated with either 6% Dextran 60,000 (Schiwa GmbH, Glandorf, FRG) or DCLHb (10 g/dl; Baxter

Healthcare Corporation, IL, USA), administered 10 min prior to the release of ischemia.

Following reperfusion, there was a significant increase of rolling and sticking leukocytes in Dextran-60 treated control animals (Fig. 5), which was associated with a significant increase in macromolecular extravasation of the plasma marker FITC-dextran 150 kD and a decrease of functional capillary density, indicated by the number of red-blood-cell-perfused capillaries per observation field. These microcirculatory disturbances were effectively attenuated in animals treated with DCLHb after reperfusion in the post-ischemic tissue. Baseline values of tpO_2 in the skin muscle ranged between 18.3–19.4 mmHg. Following reperfusion, there was a significant increase of tpO_2 in DCLHb-treated animals compared with those receiving NaCl 0.9%, while no significant differences were found compared with Dextran-60 treatment (Fig. 6).

These data show that DCLHb significantly reduces post-ischemic microvascular disturbances in striated skin muscle, which is associated with an improvement of tissue pO_2 in the early reperfusion period. The findings have been supported by electron microscopic analysis of the post-ischemic tissue, revealing a significant reduction of muscle fiber disintegration and capillary endothelial swelling. These results indicate a beneficial action of DCLHb on post-ischemic reperfusion injury in striated skin muscle.

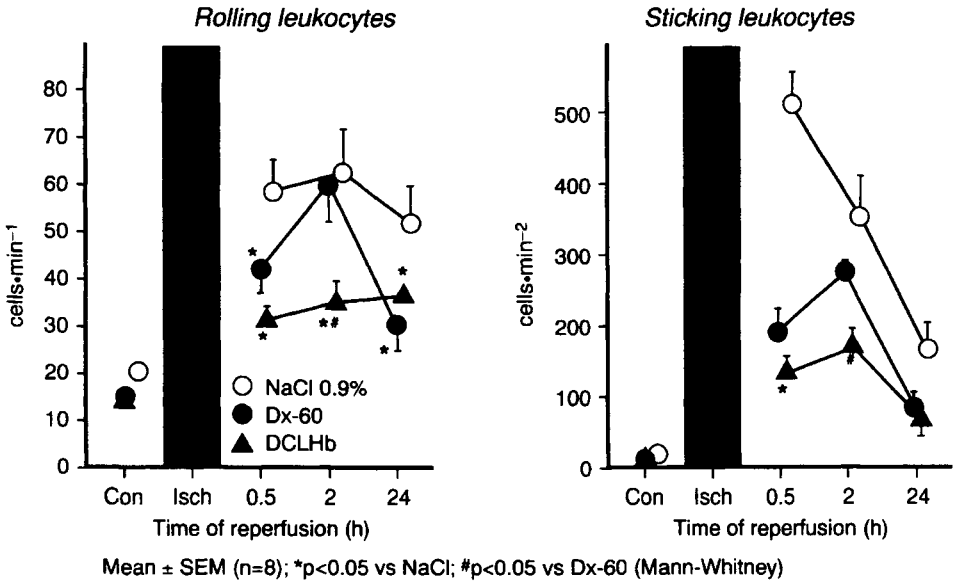

Fig. 5. Effect of Dextran 60, 0.9% saline and DCLHb on the incidence of rolling and sticking leukocytes in treated animals. A significant increase in macromolecular extravasation of the plasma marker FITC-dextran 150 kD was noted in the dextran-60-treated animals.

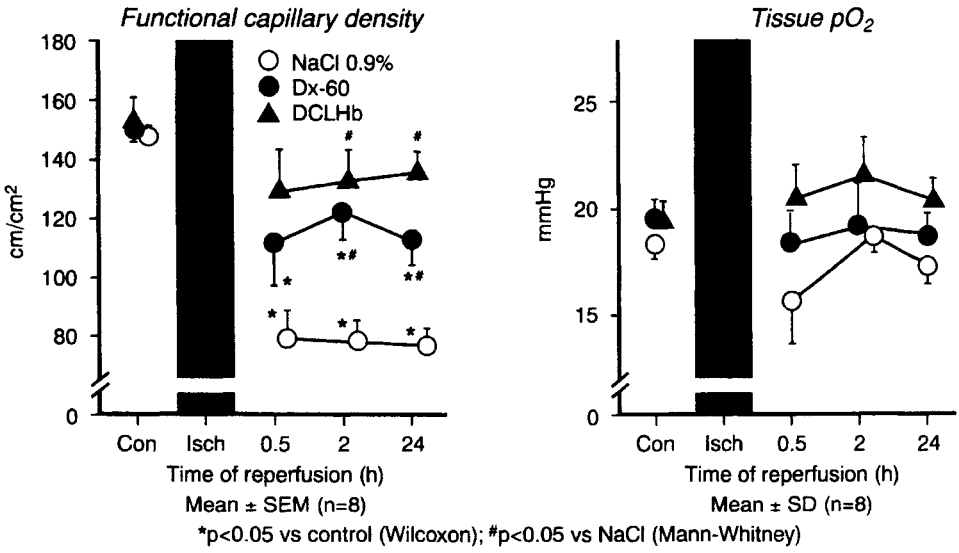

Fig. 6. Effect of Dextran-60, 0.9% saline and DCLHb on the functional capillary density and tissue pO_2. Following reperfusion there was a significant increase in tissue pO_2 in DCLHb-treated animals compared with those treated with 0.9% saline.

Microcirculatory, colloidal and vasoactive properties of DCLHb in septic rats

The objective of this study [15] was to determine the effects of DCLHb on microcirculatory blood flow in septic rats, using the ileal mucosa as the target organ of interest. This prospective, randomized study measured and compared the effects of DCLHb and Pentastarch on hemodynamics and small bowel microvascular blood flow in 24 septic rats. Twenty-four hours after creating sepsis by cecal ligation and perforation, rats were anesthetized and ventilated. The ileum was mobilized via midline abdominal incision and prepared for intravital microscopy. Baseline hemodynamic values were obtained and video microscopy was performed on 4–10 villi. Rats were then randomized to receive 2 ml of DCLHb solution (100 mg/ml, $n = 12$) or intravenous Pentastarch ($n = 12$) and measurements were repeated after 20 min. Rats treated with DCLHb then received nitroprusside to restore mean arterial pressure (MAP) to baseline levels, and final measurements were obtained 15 min later. Investigators, who were blinded to the nature of the study solution, used computerized image analysis to determine intercapillary areas (ICA, which is inversely related to capillary density).

The results indicated that ICAs were improved with both DCLHb and Pentastarch. Cardiac index was elevated by both treatments, while MAP increased only with DCLHb following an increase in systemic vascular resistance. The effect of DCLHb on capillary density was reversed by nitroprusside ($p < 0.05$, paired t-test).

In septic rats, DCLHb and Pentastarch improved capillary density and systemic

flows, but vasoconstrictor tone increased only with DCLHb. The benefit of DCLHb on blood flow and tissue oxygenation may be related to both its colloidal properties and its effects on blood pressure. Preliminary data from ongoing studies show that long-term administration of DCLHb increases tolerance against anemic hypoxia caused by hemodilution, probably as a consequence of improved microcirculatory perfusion [15].

Product-development cycle of DCLHb

The unique properties of DCLHb have lead to its potential use in a broad range of clinical applications. Figure 7 outlines the preclinical studies performed with DCLHb. Working with a variety of investigators around the world, including hematologists, cardiologists, virologists, physiologists, etc., hemoglobin therapeutics, such as DCLHb, have been found to work in a variety of indications. Despite generating extensive preclinical data on the ability of DCLHb to carry and deliver oxygen, and thereby act as a "blood substitute", DCLHb also appears to work in a

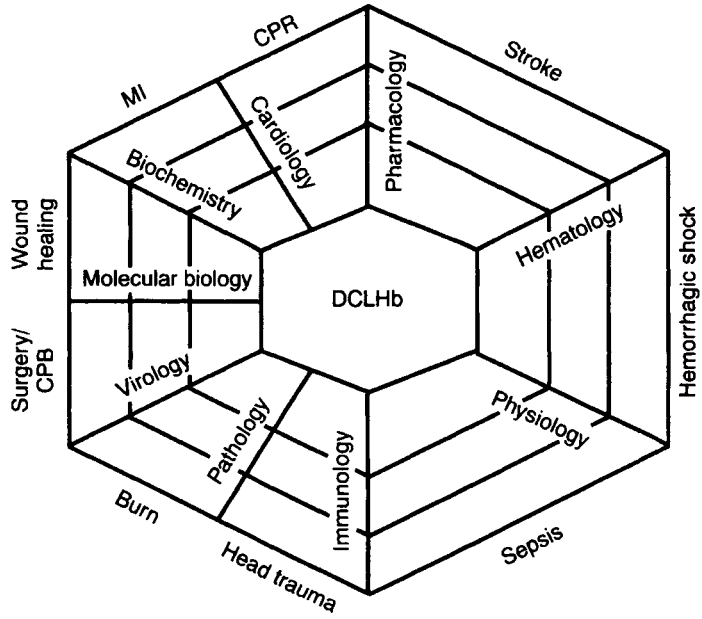

Fig. 7. Illustration of the integrated preclinical research strategy developed by the Baxter Hemoglobin Therapeutics Program ("spider-web" approach). Research is simultaneously conducted on a number of different potential clinical indications with a number of well-respected academic investigators worldwide who have a broad diversity of both basic research and clinical training and interest in different specialties. MI = Myocardial infarction; CPR = Cardiopulmonary resuscitation; CPB = Cardiopulmonary bypass.

variety of situations where blood would not be a common treatment of choice, such as in the treatment of stroke, myocardial infarction, or sepsis.

Baxter has enrolled more than 850 patients in studies to date, with more than half receiving DCLHb. As a result of the extensive preclinical testing of DCLHb and the increasing clinical experience with the product, the mechanism of action of the product is beginning to be understood. Numerous clinical trials in Europe and the USA have been completed. At present, there are three Phase III trials ongoing. Patients are being enrolled in a Phase III hemorrhagic shock trial in the USA, a European pre-hospital hemorrhagic shock trial and in a surgery/perioperative study in the USA.

An important part of the clinical development plan has also been to investigate the effects of DCLHb in a broad range of different patient populations, each with a different potential risk/benefit profile. Studies are underway to evaluate DCLHb in perioperative setting, in patients undergoing high blood loss orthopedic and aortic aneurysm repair surgeries, and in post-cardiac surgery patients. DCLHb has also been investigated in critically ill patients with sepsis, stroke patients, patients undergoing gastrointestinal surgery, and in some other unique populations. Overall, DCLHb seems to be well tolerated, both in animals and in people and, in selected patients, may result in decreased red cell requirements.

DCLHb in surgery

Clinical benefits of DCLHb in cardiac surgery. A multicenter, randomized trial in cardiac surgery patients was undertaken to determine the efficacy of DCLHb in reducing or preventing the post-operative use of blood transfusions. More than 1500 patients undergoing cardiac surgery were screened to enter this study. Of these, 209 were in need of transfusion and randomly received up to three units (total 750 ml) of 10% DCLHb or up to three units of packed red blood cells (pRBCs) within 24 h of surgery (Fig. 8). Following this 24-h period, patients could receive additional transfusion of pRBCs or other treatment, as necessary.

On the day of surgery (or through day 1), DCLHb was effective in avoiding the transfusion of pRBCs in 59% of the patients who were in need of transfusion within 24 h of surgery. At 7 d after surgery, 19% of the DCLHb-treated patients had completely avoided a transfusion of pRBCs [16]. The mean number of units of pRBCs transfused during the 48-h post-surgical period was significantly lower in DCLHb-treated patients than controls ($p < 0.05$); after this period, there was no difference between the mean number of pRBC units transfused in DCLHb and control patients.

DCLHb in general surgery. A Phase III, randomized, double-blind, pRBC-controlled trial of DCLHb is currently underway in 400 patients undergoing general surgery, including aortic repair, hip replacement, knee replacement, and abdominal/pelvic procedures. The endpoints of this study, due for completion in late 1998, are an

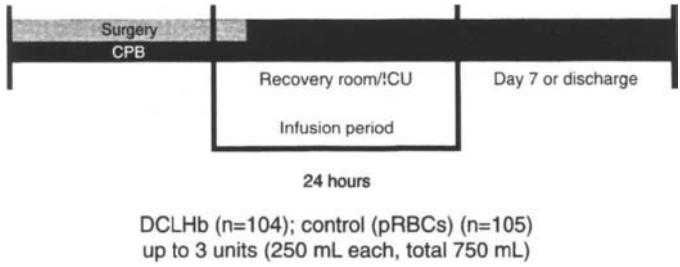

Fig. 8. The design of a study to assess the effect of post-operative DCLHb infusion on the subsequent number of units of packed red blood cells (pRBCs) infused. Two hundred and nine 209 patients were randomized to receive up to three units of 10% DCLHb ($n = 104$) or up to three units of pRBCs ($n = 105$) as control, within the first 24 h following surgery, according to clinically determined requirements. The number of subsequent units of pRBCs administered was noted in both groups.

increase in the percentage of patients avoiding pRBCs and a reduction in the total number of pRBC units used.

Potential for DCLHb in trauma/intensive care

DCLHb in critically ill patients. An interesting study involved the use of DCLHb in critically ill patients in Leeds, England. This study was a non-randomized, prospective observational pilot study, and as such the results have limited application. The study included critically ill patients who were being given maximal doses of inotropes, but were no longer responsive to all other standard ICU treatments. The majority of patients had sepsis syndrome with low vascular resistance. The objective was to observe the effects of DCLHb in this population, investigate the metabolism of DCLHb, identify the optimal administration dose of DCLHb, and to determine if there was an incremental effect of DCLHb. Informed consent was obtained from the families of all patients.

Treatment with DCLHb consisted of up to five infusions of DCLHb at half-hour intervals, with the decision to infuse being held by the investigator. Each time an infusion was deemed necessary, patients would receive a 100 ml dose of DCLHb. A total of 14 patients were enrolled. The results included a rapid vasopressor response to DCLHb and norepinephrine requirements were reduced: in some patients, administration of pressor agents was avoided completely [17]. Cardiac index and urine outflow remained constant following DCLHb infusion. The mean APACHE scores decreased significantly in the DCLHb-treated patients 24 h after treatment and gastric pHi also had a trend towards improvement.

Regarding the tolerability of DCLHb, there was a transient rise in bilirubin in two patients and a rise in AST in one patient, although the significance of these findings in this critically ill population is not known. Therefore, even in these significantly ill patients, DCLHb seemed to have a beneficial effect. In fact, five of the 14 patients

were alive at 28 d and three patients survived their septic episode and were discharged from the hospital [17].

DCLHb in the management of acute anemia. This was a randomized, double-blind, controlled study involving 23 critically ill, predominantly septic patients; those with a hemoglobin level of 8–9 g/dl received either 1 unit (250 ml) of 10% DCLHb or 1 unit of pRBCs; patients with a hemoglobin level of <8 d/dl received either 2 units (500 ml) of DCLHb or 2 units of pRBCs. Oxygen delivery (DO_2), oxygen consumption (VO_2), as well as overall organ function and metabolic status were determined at various times throughout the following 24 h. The oxygen extraction ration (O_2ER) was used to assess oxygen utilization by tissues.

The results indicated that DCLHb was well tolerated in acutely anemic, critically ill patients, improving consumption of oxygen by tissues compared with fresh (<2 weeks old) pRBCs, despite a decrease in calculated global DO_2 [18]. In addition, DCLHb allowed the avoidance of pRBCs for at least 48 h after treatment.

DCLHb in traumatic, hemorrhagic shock. Perhaps of more interest than the use of DCLHb as a traditional "blood substitute", is its potential use in traumatic hemorrhagic shock. This is an important public health problem with high mortality and advances in the current standard of care are needed.

Current resuscitation, at least in the pre-hospital setting in the USA, primarily involves the infusion of large volumes of crystalloid solutions. However, recent studies by Bickell et al. [19] have questioned the use of large volumes of lactated Ringer's, which may actually increase bleeding, decrease normal clotting time, and result in decreased tissue oxygen delivery and increased mortality. In addition, infusion of these large volumes of solution requires optimal intravenous access. The alternative is infusion of O-negative or type-specific blood, although both of these options have associated risks and are not usually available at the accident scene. Available pressor agents, which cause vasoconstriction without affecting vascular volume or an increase in oxygen delivery to tissues, have limited use, particularly in patients who present with low vascular volumes.

Therefore, the approach to investigate solutions such as DCLHb in this setting is based on the hypothesis that oxygen carriers, given very early at the accident scene, should result in less fluid requirements and may result in a shorter duration of ischemia, leading to less of an inflammatory response, preservation of cellular organ function, and hopefully, less ultimate cell death, organ failure and decreased mortality. If, as shown in animal studies, DCLHb can redistribute blood flow and oxygen to vital organs, reduce bacterial translocation and sepsis, increase the homogeneity of oxygenation in the tissues, as well as replace lost vascular volume, infusion of the product should reduce morbidity and mortality and may allow the better use of existing blood supplies, and in some situations, serve as a bridge to transfusion.

Two Phase III, randomized, controlled trials of DCLHb in 1000–1500 patients in

severe traumatic hemorrhagic shock are currently underway in Europe and the USA. In the European trial, approximately 500 patients will receive up to 1000 ml of DCLHb or standard care "on scene", with endpoints being a reduction in morbidity (organ dysfunction) and 28-d mortality. The US trial will enroll at least 800 patients who will receive either DCLHb (up to 1000 ml) or standard care "in hospital", with the primary endpoint being a reduction in 28-d mortality

DCLHb in other patient populations

DCLHb in hemodialysis patients. A unique study conducted with DCLHb involved infusion of low doses of DCLHb in patients undergoing hemodialysis [20]. The rationale for this study was to assess the safety of DCLHb and determine its pharmacokinetics in this patient population. Another goal was to determine if the DCLHb pressor effect may provide any potential benefits, since hypotensive events are common during hemodialysis. Currently, the course of treatment for these unexpected events is to infuse boluses of hypertonic saline to restore blood pressure to baseline values. Unfortunately, infusion of the hypertonic saline ultimately negates much of the benefit of fluid removal obtained during dialysis.

The study had a randomized, single-blinded crossover design in which patients received either small doses of saline or DCLHb. In this setting, the DCLHb was not used as a blood substitute, but rather, as a hemoglobin therapeutic. DCLHb or saline was infused over a 30-min period at the start of dialysis, and each subject in the crossover design served as his or her own control. There were six patients per dose.

The results of this study showed that DCLHb infusion resulted in better blood-pressure stabilization. The number of hypertonic saline interventions required in the normal saline group was 20 in nine patients, whereas only one intervention was required in one patient receiving DCLHb.

DCLHb in stroke. DCLHb is also being studied in stroke. In animal models of cerebral ischemia, DCLHb results in a significant reduction of the infarct size and the duration of the ischemia may be extended in the presence of DCLHb.

Summary

These various potential applications of DCLHb may be classified into three broad categories:

1. As a red blood cell substitute — DCLHb may be used as blood, taking advantage of its volume expanding and oxygen-transport properties. In this category, Baxter is involved in studies with blood equivalency, general surgery, and cardiac surgery
2. As a hemoglobin therapeutic — Baxter is or has been involved in clinical trials investigating the use of DCLHb in hemodialysis, stroke, aortic aneurysm repair, enhanced blood pressure stability during orthopedic and gastrointestinal (GI)

surgery, and other situations where the pressor properties of DCLHb may be useful

3. In between these two extremes — DCLHb is being investigated as a potential agent for enhancement of perfusion. The major indications include hemorrhagic shock and critically ill patients. Overall, as a result of the variety of properties of this agent, DCLHb is being evaluated across a broad spectrum of potential clinical applications.

The future for hemoglobin therapeutics

At least six companies are currently involved in research into the use of hemoglobin therapeutics. Each company is developing a unique product, some using human blood, some using animal blood and others using recombinant technology. Likewise, each company has a particular approach to intermolecularly and/or intramolecularly crosslinking its product.

Regarding the future of this field, it is important to consider that almost all of these companies' products are in human clinical trials. Many of these companies have completed the necessary animal testing to allow entrance into human testing and, in many cases, have also passed the initial safety hurdles of Phase I testing and are at more advanced stages of testing.

Many in the field of hemoglobin therapeutics are optimistic that the final picture will include market approval of these products. If proven effective, these solutions should represent an exciting new class of therapeutic agents for potential use in a wide variety of clinical applications.

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CHAPTER 7

Update on Perfluorocarbon-Based Oxygen Delivery Systems

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Introduction

Few options are given to the critical care physician to combat tissue hypoxia and its consequences. Once circulating volume has been restored and the patient has been given oxygen to breathe, the only recourse presently available is to transfuse blood. Transfusion is not without consequences, however, and the benefit of transfusing stored blood has been questioned. Hence the intense efforts that are being devoted to finding an alternative to transfusion for relieving an oxygen deficit and restoring compromised physiological indicators in the surgical and critical care patient. The ideal anti-hypoxic drug is, by essence, oxygen. The challenge is to deliver this drug rapidly at the sites where it is needed. An effective oxygen delivery system could also be used to *prevent* occurrence of ischemic events and could revolutionize standard patient care.

A synthetic carrier solvent for oxygen

Parenteral administration of oxygen requires that the gas be formulated in a compatible liquid form. This requires the use of either a carrier molecule to which the oxygen molecule is coordinated (chemically bound), as is the case for hemoglobin, or a passive carrier solvent in which oxygen is physically dissolved. The most effective solvents known for gases, including oxygen and carbon dioxide, are perfluorocarbons [1].

Perfluorocarbons (PFCs, or perfluorochemicals, or fluorocarbons) are synthetic organic compounds in which all or most of the hydrogen atoms have been replaced with fluorine atoms. Fluorines are larger and much more electronegative than hydrogens. The fluorine-carbon bond is the strongest encountered in organic chemistry. Fluorocarbon moieties are protected against reagents by a dense, repellent sheath of electrons. On the other hand, the interactions that exist among fluorocarbon molecules are much weaker than those present among hydrocarbon molecules. This set of attributes results in dramatic differences in properties between fluorocarbons

and hydrocarbons. Liquid fluorocarbons, and highly fluorinated materials in general, have exceptional thermal and chemical stability; are simultaneously hydrophobic and lipophobic (i.e. repel both water and lipids); have non-adherence and anti-friction properties; and display unique dielectric, rheologic and optical characteristics. The unrivaled gas dissolving capacity of liquid fluorocarbons is a consequence of their weak intermolecular forces, which facilitate the insertion of gas molecules. Compared to hydrocarbon analogs, PFCs also have greater fluidity, lower surface tensions, higher densities, magnetic susceptibilities closer to water, and tend to spread more readily on aqueous saline surfaces. Highly fluorinated materials have multiple benefits for the chemical, electronic, nuclear, communication, and aerospace industries.

In the biomedical arena, neat PFCs are in clinical trials utilizing liquid ventilation for treatment of acute respiratory failure [2,3]. A neat fluorocarbon has been approved for oral use as a diagnostic contrast agent [4]. Fluorocarbons are used in ophthalmology for retinal manipulation during vitreoretinal surgery [5]. Aqueous dispersions of microbubbles comprised of fluorocarbon-containing gaseous mixtures are being evaluated as contrast agents for ultrasound imaging [6,7].

For intravascular use, PFCs must be emulsified, i.e. dispersed into small, sub-micron-size droplets in a physiological aqueous electrolyte solution. Each PFC droplet is coated with a thin film of a surfactant (an amphiphilic molecule that combines hydrophilic and hydrophobic moieties), which reduces the large surface tension (50–60 $mN\,m^{-1}$) that exists at the fluorocarbon/water interface, and serves both as an emulsifier and an emulsion stabilizer [8]. References 8–20 of this chapter include recent literature sources in the area of fluorocarbon emulsions for oxygen delivery.

Further fluorocarbon-based systems under investigation include reverse (i.e. water-in-fluorocarbon) emulsions, multiple emulsions, microemulsions, gels, vesicles, tubules, and other colloidal systems. Their potential use is for the delivery of drugs (including oxygen, nitric oxide, etc.) and other active agents [9].

The "physiology" of PFC emulsions

PFCs do not mimic hemoglobin. They do not bind oxygen, but dissolve it (similar to water, only 20 times more effectively). Oxygen dissolution in a PFC is directly proportional to the partial pressure of the gas. Oxygen concentration in a fluorocarbon emulsion can therefore be increased up to five-fold by just increasing the oxygen fraction (FiO_2) in the air inspired by the patient. Oxygen release from a PFC does not depend on an allosteric effector. Contrary to stored red blood cells which take 6–12 h to deliver oxygen effectively due to deterioration (including loss of 2,3-diphosphoglycerate [2,3-DPG]) during storage, release of oxygen from PFCs is immediate. Due to the absence of chemical bonding to the carrier molecule, the dissolved oxygen is readily available. Oxygen is extracted very effectively from PFCs and extraction typically reaches 90% of the dissolved oxygen, compared to 25–30%

for hemoglobin in standard conditions. While hemoglobin releases oxygen less efficiently when temperature is decreased (a situation which is further aggravated by an increase in blood viscosity), fluorocarbons are somewhat more effective due to slightly higher gas solubility at lower temperatures.

Infusing a PFC emulsion in the vasculature is to a large extent equivalent to increasing the solubility of oxygen in the plasma compartment of blood. High FiO_2 results in a large oxygen tension gradient, providing an effective driving force for oxygen diffusion. The fact that the PFC droplets are very numerous and very small appears to further facilitate oxygen diffusion between red blood cells and tissues. These mechanisms are expected to be most effective in the capillary beds, especially in hemodiluted patients [10].

Due to their submicron size, PFC emulsion particles are postulated to flow mainly in the thin plasma layer between the red blood cells and the vessel wall of the larger blood vessels and arterioles (near-wall particle excess phenomenon). In the microcirculation the PFC emulsion particles will occupy the large plasma gaps between red blood cells and can easily perfuse all of the capillaries in the microcirculation under all conditions. Even when localized vasoconstriction or ischemia prevent normal perfusion with red blood cells, there will always be some plasma flow to transport the PFC emulsion particles through these tissues. The largest plasma gaps between red blood cells will occur in the microcirculation. Consequently, this is where the addition of PFC emulsion particles may have the greatest benefit, i.e. by providing additional oxygen delivery to tissues and thereby preventing tissue hypoxia. PFCs load and unload oxygen about twice as fast as hemoglobin since they exchange gases by simple diffusion.

These characteristics of PFC emulsion particles contribute to the remarkable ability of PFC emulsions to enhance tissue oxygenation even when the total dose administered does not appear to be large enough to significantly increase arterial oxygen content (based on simple calculations which take into account only the oxygen carrying-capacity of the PFC).

Fluorocarbon droplets are progressively removed from the circulation by phagocytosis. This mechanism is responsible for the limited intravascular persistence of the emulsion. The PFC is then stored temporarily in the reticuloendothelial system (RES) organs, progressively returned to the circulation in dissolved form by lipid carriers, and eventually excreted with expired air through the lungs [11]. The half-life of emulsion droplets in the circulation is typically in the 4–15 h range, depending on particle size and dose. Half-lives of PFCs in the RES depend primarily, and exponentially, on molecular weight. Retention in the RES can, however, be favorably influenced by conferring some lypophilic character on the PFC [8].

Two transient biological side-effects have been reported in clinical studies following infusion of PFC emulsions: (1) a short-lived febrile response 4–6 h after dosing, and (2) a transient drop in platelet counts at 2–3 d post-dosing (but with no effect on platelet function or bleeding time). The mechanisms for both of these PFC emulsion-related effects have been elucidated, and are related to the normal clearance of the tiny

emulsion particles (< 0.2 μm) from the circulation by the phagocytic cells of the RES (Kupffer cells in the liver and macrophages in the spleen) and to the physical properties of the emulsion (particularly particle size and choice of surfactant) [11].

These side-effects have been minimized by reducing particle size and tightly controlling particle size distribution [8]. No hemodynamic changes have been observed with the present egg yolk phospholipid (EYP)-based perflubron emulsion formulations. No effect on pulmonary function was observed in humans [11].

Fluorocarbons are not metabolized; therefore, metabolite-related toxicities are not an issue. PFCs do not leak out of the vasculature, do not inactivate endothelial-cell-derived nitric oxide, and do not prevent the increase in cardiac output that normally occurs following hemodilution. They are not subject to oxidation and do not participate in free radical reactions in vivo. No bacteria are known to feed on fluorocarbons and there has been no report of enzymatic cleavage. It should also be noted that the emulsions have good mechanical resistance to pumps and filters.

Key advantages of PFC emulsions in comparison to hemoglobin-based products include higher oxygen extraction, absence of vasoconstrictive effects, an unrestricted supply, and cost-effective, large-scale production capability.

Progress in fluorocarbon emulsion technology

The first generation emulsions, of which Fluosol® (Green Cross Corp., Osaka, Japan) was the archetype, demonstrated effective, though temporary, oxygen delivery [12–14]. These emulsions, however, had several limitations including poor stability, short intravascular performance, side-effects related to complement activation, long organ-retention of one of the fluorocarbon components, excessive dilution, need for frozen shipment and storage, and a cumbersome reconstitution procedure. The indication proposed for Fluosol, anemia, and a strategy for use incompatible with the product's short circulation life led to an initial rejection by the United States Food and Drug Administration. Approval was gained subsequently for a more realistic indication, for use of Fluosol in conjunction with percutaneous transluminal coronary angioplasty (PTCA); however, lack of user-friendliness and the development of autoperfusion catheters impeded the product's commercial success.

The evolution of emulsion formulation and characteristics since the development of Fluosol is characterized by at least six significant improvements [8,15], including:

1. The selection of fluorocarbons with some lipophilic character, since lipophilicity has been found to facilitate the PFC's excretion from tissues while also contributing to stabilizing the emulsion.
2. The poloxamer (Pluronic® F-68) utilized as the emulsifier in Fluosol was replaced by EYP, which produces more stable emulsions, especially when combined with lipophilic fluorocarbons. EYP does not cause the complement activation-related side-effects associated with Pluronic. In addition, these phospholipids have a long

history of medical use in lipid emulsions for parenteral nutrition and they are key components of liposomes that are being investigated for drug delivery.
3. The PFC concentration was increased several fold, resulting in increased oxygen-carrying capacity for a given volume of emulsion, as well as increased versatility.
4. The overall emulsion formulation was simplified; the salts no longer need to be separated from the stem emulsion.
5. The new emulsions are significantly more stable; they no longer need to be frozen for storage and shipment, and they have a shelf-life of approximately two years at standard refrigeration temperatures.
6. The complex reconstitution procedure from a frozen stem emulsion and two annex solutions that impeded Fluosol has been eliminated; the new emulsions are ready for use.

Needless to say, these improvements were paralleled by a significant augmentation of our knowledge of the physical chemistry and in vivo behavior of fluorocarbon emulsions.

Selecting a fluorocarbon that has all the desirable features for in vivo administration and achieving emulsion stability compatible with widespread use took well over a decade. Perfluorooctyl bromide (perflubron) stands out among the candidate fluorocarbons, as it appears to have the right degree of lipophilicity to ensure rapid excretion (half-life of ca. 4 d in RES tissues for a 4 g/kg dose). Its molecular weight (499) ensures low volatility, thus minimizing the risk of interference with lung physiology. Its lipophilic character also leads to a good match with phospholipids from the point of view of emulsion stability. Perflubron has approximately a 25% higher O_2-dissolving capacity than perfluorodecalin and perfluorotripropylamine, the fluorocarbons utilized in Fluosol. Last but not least, perflubron can be cost-efficiently produced in high purity on a large scale.

Achieving emulsion stability was another major task which is essential for a commercially viable product [8,16]. Particle size increase over time in submicron fluorocarbon-in-water emulsions was shown to result from an Ostwald ripening process by which individual PFC molecules leave the smaller droplets, where the chemical potential is larger (as a consequence of the Kelvin effect), diffuse through the aqueous phase, and rally the larger droplets. The process can be slowed down by reducing the interfacial tension between the PFC and water, and by reducing the solubility and diffusibility of the PFC in water. Phospholipids have proved very effective in reducing the perflubron/water interfacial tension [17]. PFC solubility and diffusibility can be diminished by adding a higher molecular weight PFC component, although heavier PFCs generally have longer organ retention times. This can, however, be circumvented by using a lipophilic heavier PFC [18]. Another approach to fluorocarbon emulsion stabilization involves the use of mixed fluorocarbon-hydrocarbon diblock compounds that are believed to concentrate at the interface between the fluorocarbon and the phospholipid layer that surrounds them [19].

The most advanced of the present generation of PFC emulsion products, consists

of a 60% w/v (~ 32% v/v) concentrated emulsion of lipophilic perfluoroalkyl bromides (Oxygent™, AF0144, developed by Alliance Pharmaceutical Corp., San Diego, CA). The primary fluorocarbon is perfluorooctyl bromide. A small amount of its higher homologue, perfluorodecyl bromide, is added for stabilization against molecular diffusion. EYP serves as the only emulsifier. The aqueous phase consists of buffered saline to maintain pH and osmolality control. Minute amounts of an antioxidant, tocopherol, and of a chelating agent, EDTA, are added to protect the EYP against oxidation. Emulsification is achieved using high pressure homogenization devices. The product is terminally heat sterilized above 120°C in a rotary autoclave. It can be stockpiled under refrigeration for about two years.

Recent formulation adjustments and process optimization efforts have led to small, narrowly dispersed and well controlled particle sizes averaging 0.16 ± 0.01 μm in diameter, resulting in a substantial reduction in biological side-effects [8,20]. The flu-like symptoms observed with the earlier formulations occur less frequently, and the reduction in platelet counts has been attenuated. The perflubron emulsion is, of course, compatible with all blood types and does not transmit blood-born infectious diseases.

Another EYP-based emulsion, Oxyfluor (HemaGen-PFC, St Louis, MO), is also undergoing clinical testing [21]. Oxyfluor utilizes α,ω-dichloroperfluorooctane as the PFC and has a somewhat larger particle size. A product close to Fluosol in its formulation (except for the use of a heavier perfluoroamine, perfluoro-*N*-methylcyclohexylpiperidine, instead of perfluorotripropylamine, and a somewhat different poloxamer), Perftoran (Perftoran Co, Pushchino, Russia), has recently been approved for a variety of uses in Russia [22].

Commercial scale production

Perflubron is just one step away from a key industrial intermediate, perfluorooctyl iodide, on the production line that leads to polytetrafluoroethylene (Teflon®) and to major industrial fluorosurfactants. This intermediate is produced by a telomerization process that ensures a high degree of purity. Industrial capacity for manufacturing 99.9% pure perflubron on a 100 ton per year scale already exists. Technology for large scale GMP production and sterilization of PFC emulsions is now also well established. An automated, validated facility has been built by Alliance Pharmaceutical that can produce and package about 1 million clinical doses of perflubron emulsion per year.

Target indications

Effectiveness of fluorocarbon emulsions in delivering oxygen is at a maximum when hemoglobin levels are low and cardiac output is elevated. One of the target indications that takes into account both this characteristic and the PFC's short intravascular

half-life is their use during surgery in combination with acute normovolemic hemodilution (ANH) [12,13,23–28]. This practice is expected to result in increased patient safety by preventing tissue hypoxia, and in reducing the need for donor blood transfusions. The breadth of this application is indicated by the fact that about 60% of red blood cells transfusions occur in the perioperative period.

Extensive preclinical experimentation has demonstrated that PFC-based oxygen carriers are capable of delivering the amount of oxygen predicted by computer calculations and of improving mixed venous oxygen tension and tissue oxygenation [25,27], (see also [23,29–36], for example). Nonclinical studies in canine models of profound hemodilution, designed to mimic acute surgical anemia and blood loss, have, for example, confirmed that perflubron emulsion can prevent tissue hypoxia (brain, heart, gut, liver, and muscle) and preserve myocardial and cerebral function. The possibility of practicing deeper hemodilution and the potential for allogeneic blood transfusion reduction has also been demonstrated.

Clinical development

To date, a total of 17 different clinical studies with perflubron-based emulsions have been completed and have enrolled 540 subjects (340 have received perflubron emulsion, 65 were randomized to receive a unit of autologous blood, and 135 were volume-matched placebo controls).

Safety studies

Extensive studies in over 200 healthy volunteers and surgical patients have clearly established the safety of perflubron emulsions. Two supplemental safety studies with the current 60% w/v perflubron-based emulsion (AF0144) were conducted with 48 volunteers (24 male, 24 female) to specifically focus on hemostasis and immune function parameters. These studies established the absence of any direct effects on platelet function (based on ex vivo aggregation assays), template bleeding times, and coagulation parameters (PT, PTT, and fibrinogen). In addition, there was no evidence of any complement activation or immunogenic reactions; no suppression of humoral or cell-mediated immune function; no abnormal changes in liver, pulmonary, or renal function; no clinically meaningful effect on blood chemistry; and no hemodynamic effects or vasoconstriction.

Compared to previous studies with earlier PFC emulsions, the incidence and magnitude of the delayed febrile response has been substantially reduced with AF0144. A small, transient increase in body temperature (1°C to 2°C) was observed in only 5 of 32 perflubron emulsion-treated subjects. In addition, the mild decrease in platelet count (< 20% from baseline level at 3 d post-dosing with AF0144) observed only in the high dose group (1.8 g PFC/kg) was less than previously reported for other PFC emulsion formulations.

Pharmacokinetic analysis of blood perflubron levels in these studies indicated that

the circulating blood half-life for perflubron emulsion was dose-dependent, i.e., 6.1 ± 1.9 h at a dose of 1.2 g PFC/kg and 9.4 ± 2.2 h at a dose of 1.8 g PFC/kg. Current clinical studies in elective surgery patients are employing doses up to 2.7 g PFC/kg.

Activity and efficacy studies

Earlier 1997, two multicenter, randomized, controlled single-blind Phase IIb studies with AF0144 were completed. A total of 256 general surgery patients were enrolled, including 147 orthopedic patients in the European study and 109 urological and gynecological patients in the US study. The main study objectives were to demonstrate safety in surgical patients, and to evaluate AF0144 as a temporary oxygen carrier versus autologous blood in reversing "transfusion triggers" (i.e. the normal physiological parameters that mandate the need for a blood transfusion). Transfusion trigger parameters employed in these studies included predefined increases in cardiac output and heart rate, decreases in mean arterial pressure and mixed venous pO_2, any changes in ECG and/or ST segments indicating myocardial ischemia, and a minimum Hb level. Results of these two studies indicated that with respect to safety, there were no serious adverse events associated with the i.v. administration of perflubron emulsion and there were no clinically significant effects on hemodynamic parameters, hematology, or blood chemistry. With respect to efficacy, the primary endpoints were achieved in both studies. Perflubron emulsion was found to be more effective than fresh autologous blood at reversing transfusion triggers, and delayed the need for subsequent blood transfusion [37].

Alliance's early concept that relatively small doses of an appropriately designed fluorocarbon emulsion, when administered to hemodiluted, oxygen-breathing patients, could be more effective than blood in improving tissue oxygenation (as demonstrated by reversal of physiological indicators for transfusion) appears to be substantiated. When combined with ANH and other blood saving strategies, the oxygen carrier may allow more aggressive hemodilution to be practiced without risk for the patient, and provides a potential new strategy for reducing allogeneic blood transfusions during surgery. It may therefore also contribute to promoting ANH.

Fluorocarbon-based oxygen carriers may also prove useful in cardiopulmonary bypass (CPB) surgery where they could play a dual role: first as an oxygen-carrier added to the priming solution in the extracorporeal circuit, thus improving oxygen supply and potentially reducing the need for allogeneic red blood cells [31] and, second, by dissolving the tiny air bubbles that may be introduced into the circuit and can cause microemboli, which can lead to post-surgical neurological dysfunction [38].

Three Phase II studies using perflubron emulsion AF0144 in cardiac surgery have recently been completed. Approximately 80 patients undergoing CPB were enrolled. One study focused on evaluating cerebral and systemic oxygenation status and neurobehavioral outcome post-CPB, while the other study was designed to determine the minimum acceptable hematocrit on bypass following autologous blood harvesting. The third Phase II study was to assess the blood pharmacokinetics of perflubron

emulsion following different dosing regimens. Ongoing data analysis indicates that AF0144 was well tolerated, and demonstrated the potential for improving systemic oxygenation status and reducing the need for allogeneic blood transfusion. Pivotal Phase III clinical studies are expected to begin soon.

Another important situation in which PFC-based oxygen carriers should prove beneficial is trauma, especially during the pre-hospital period, the "golden hour" which has a large impact on outcome for the patient, and during which blood is usually not available. They may also be useful in the hours that follow transfusion, during which stored blood has not yet reached its full effectiveness.

More generally, fluorocarbon emulsions could be used to provide oxygen systemically to surgical patients at risk of tissue hypoxia or ischemia. These products could also help reduce the number of one-unit transfusions that are given as a result of uncertainty as to the exact status of an unstable patient. Administration of the emulsion would provide insurance to the patient, comfort to the physician, and defer the decision of whether or not to transfuse until the patient's status is properly assessed.

Potential clinical indications for the perioperative use of PFC emulsions include:

1. protecting the tissues from acute ischemia (e.g. stroke, myocardial infarct) or temporary oxygen deficit due to transient anemia (e.g. trauma, surgical blood loss);
2. protecting the brain from gaseous air emboli and thereby decreasing the cognitive function deficits commonly observed following CPB;
3. avoiding allogeneic blood transfusion (especially in situations when blood is not available immediately);
4. enhancing the oxygenation levels in tumors to make them more susceptible to radiation treatment and chemotherapy; and
5. preservation of organs and tissues harvested for future transplantation.

In summary, fluorocarbon emulsions provide a simple and elegant, efficacious and immediate, safe and cost-effective means of temporarily increasing oxygen delivery to tissues. Key areas of use have been identified for which at least one product, a perflubron emulsion, is in advanced clinical trials. Other potential applications, for which different formulations may be required, are under investigation.

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 8

Red Cell Substitutes: Past Problems, Current Dilemmas

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Introduction

Few can argue the desire to have a shelf storable, oxygen carrying, crystalloid-colloid solution for use in emergency situations when blood, or red blood cells, is not available. This principle has been proposed for a very long time, almost as long as the function of hemoglobin has been known — nearly 100 years. The concept of providing a red cell substitute has been around for a long time. The critical question of how to accomplish it has been the vexing and complicated if not frustrating problem. This author has been involved for nearly a quarter of a century in all aspects of the development of hemoglobin based red cell substitutes from the design of molecular modification through Phase I clinical testing, with many interesting and challenging side trips and inquiries along the way. Thus, the perspective of this piece is both personal and historical in the context of defining a philosophical basis for some of the current problems, some quite obviously rooted in the past.

It is interesting to ask: how have the ideas of science and the products of technology influenced the current product development? There is an assumed need for a red cell substitute — note the term "blood substitute" is not preferred — and whether real or just perceived, the development has proceeded apace. The precise influences on the development are complex and not easily dissected from the whole for they are complex and quite intertwined. What is clear, however, is a separation of intellectual concepts from practical reality, the latter required to effect bringing the concepts to application, the ultimate goal of the process.

Historical context

At the outset it must be appreciated that little in the way of product development occurs in a vacuum. There are always multiple and often competing reasons why a particular development has or has not reached completion. One of the obvious explanations is the science of the time is used to explain the observations. Thus, if all one has is light microscopy to investigate the effects of an intervention or experiment,

then the explanations of the observations are explained in the science of that field. As the scientific knowledge base expands — and it has exploded in the latter half of the 20th Century — more sophisticated explanations of the observations are possible. Now, instead of just observational explanations it is possible to ascribe functional reasons to the events observed by applying different levels of physiology. The explanations may now be expressed as variations in physiology at the system, organ, tissue or sub-cellular level. This clearly adds a level of complexity to the process and raises the issue of relevance for the observation, the solution and the real knowledge of the underlying physiology in health and disease. More critically, it raises the question of how to extrapolate from the microphysiology to the clinical scenario in a meaningful way. That is not meant to imply the absence of a relationship between the microphysiology and the gross values dealt with in the clinical situation. Rather, it is noted to emphasize that the microphysiology may not be reflected in the clinical picture for other compensatory mechanisms can be called upon to minimize the perturbation caused by the red cell substitute at the cellular or sub-cellular level. The theories that address complexity and system interaction are most relevant yet rarely invoked in any of the analysis used to design or evaluate red cell substitutes. This then leads to partial explanations and could direct development down some interesting and perhaps unrealistic or unproductive pathways, diverting the focus of the development. That there have been many blind alleys in the path towards a clinically useful red cell substitute must be accepted. The impact on the field in general has likely been limited and generally of no great consequence.

The transfusion trigger

Vital in the development of any red cell substitute is the establishment of a threshold for when transfusions of red cells are indicated. Unfortunately, there is little agreement on this issue. The identification of a true "transfusion trigger" has been elusive for a long time [1]. Without a reference frame for *indicated transfusions* it may be impossible to evaluate the effect or efficacy of any proposed substitute, further complicating any impact analysis. The use of blood and blood products continues to be highly variable and is expected to remain so until uniform acceptable guidelines — scientifically based — are in use [2,3]. The only generally agreed upon issue is that an absolute value of hemoglobin and/or hematocrit is not acceptable as the definition of a transfusion trigger. The decision to transfuse is an individual one and is not based on arbitrary values derived from observational studies done over 50 years ago. The modern practice of surgery and medicine generally is more attuned to a precision in decision making and will not accept undocumented values upon which to make a decision. Of course, the perceived risk of blood and blood product transfusion has driven physicians to invoke alternative measures prior to transfusion. These measures involve applying the therapeutics of oxygen delivery physiology directly to patient care and require an appreciation of that complex system — or set of systems — to

effect the desired benefit, often without the use of transfusion. That transfusion avoidance is an operational principle of modern medical care is now accepted. The perceived risks of transfusion have altered the practice [4,5].

Red cell substitutes

The history of red cell substitutes is by itself a most interesting and fascinating study. With the power of retrospective analysis it is possible to establish five distinct periods of development. Each of these periods is distinguished by the technologic advances, applied to the new concepts or ideas, that enabled the process to move forward. There is little doubt that those who came before us knew what to do and what the generic problems would be; it is clear they did not have the capability to accomplish the goals because the technology lagged behind the needs, hampering progress.

Prior to 1967 most of the information and data is of historical interest and not particularly relevant to the developments today except in one very special way. If some negative data entered the literature that indicated the toxicity of hemoglobin on a particular organ or system — independent of the study design or quality of materials used compared to the solutions of today — the ability of modern science to purge that concept and change the perception is most difficult. There are those that still consider hemoglobin nephrotoxic based on observational studies of a diverse and complex form where the end points were not physiologic nor the solutions used anywhere near as well characterized as those in various stages of clinical testing as of this writing. Old shibboleths are difficult to dislodge despite the rapidly expanding body of knowledge and clinical testing that has failed to demonstrate renal toxicity.

The current era, one of clinical testing and evaluation, has brought new and important insight into the field of red cell substitutes. It is opening up new avenues of investigation and resolving old problems while raising new issues. This is an exciting era for it affords an opportunity to integrate the modern medical approach, with its scientific basis, and the practical issues of transfusion therapy in a meaningful and useful manner. As the properties of these new solutions unfold, after all it is difficult to predict all the effects, newer applications will no doubt emerge. Those newer applications will be the result of observations made during the expanded use of the red cell substitutes, possibly leading to new insights into the global physiology so critical to clinical medicine. Whether there will be a spin off from the clinical applications to a more in-depth appreciation of the microphysiology is not yet clear.

The solutions in clinical testing are all more pure and better characterized than most of the solutions used between 1967 and the early 1990s. The very fact that they have been approved for some level of clinical testing is significant and implies an applied minimum manufacturing and testing standard that was not possible by individual laboratories. Just how much of the variation and differences in outcome for the many laboratory studies done on diverse solutions can be attributed to differences in the underling solution purity and composition may never be known.

What is important is to recognize these differences exist and could explain some of the observations — apart from the physiology — and the apparent ill effects. Once recognized as an adverse event or possible detrimental physiologic consequence attributable to the solution and not the class of solutions, pursuit of appropriate options is more likely. If any given event is perceived as the result of the class of agents, it may be most difficult to explain the observations to an acceptable level for purposes of proceeding forward. Stated explicitly, not all solutions are the same and one cannot expect the same result from the entire class of agents for the compositional and structural differences are real.

Early in the modern era of red cell substitute development a perception of an altered oxyhemoglobin dissociation curve (a shift to the left for stripped hemoglobin) as bad was proposed. It was also thought that the intravascular half life was too short and needed to be increased. Since these issues were espoused great effort has been directed to chemical modification of hemoglobin to improve its oxygen off-loading capability and increase the intravascular retention time [6–12]. The list of modifications, the modifiers and approaches taken is by itself a revealing study of the pursuit of a clinically useful product. Whether the drive is scientific, altruistic or commercial is not really relevant to the question. That effort is being expended is the more important issue for without that effort there will be no product.

It clearly takes some time for the developments to achieve clinical testing. The current products are, conceptually, 15–20 years old in design and concept. Newer products and modifications may be more useful or more practical. Exactly how these will be evaluated and what role the newer generations of materials will play is undecided for now. The newer solutions, different modifications, encapsulation, and complex formulations address more than oxygen delivery and play in some ways on the expanded knowledge base of the physiology of hypovolemic shock, sepsis or reperfusion injury. That is not to imply this is a wrong or dead-end approach. Rather, it is to imply that the fruits of the investigations into the physiology of illness can be used to direct the development of applications with an even greater potential for use.

Dependency on oxygen

It is an undeniable fact, humans are obligate aerobic organisms! Thus, the provision of an adequate supply of oxygen to the tissues — tissue perfusion — is paramount for any red cell substitute. Blood provides for a least five functions and oxygen delivery and maintenance of vascular volume are two very critical ones among the many. Maintaining adequate oxygen delivery to tissues is of prime importance in the clinical practice of medicine, especially so in the areas of trauma, critical care and the operating room. Thus, this essential property of a red cell substitute — the ability to carry and deliver oxygen — is critical for any evaluation of any proposed product. This is a basic and undeniable issue. Proving the delivery of oxygen and efficacy of a solution may not however, be quite that simple for the complexity of experimental

design coupled with the compositional differences in the solutions resulting in different characteristics must all be accounted for in any final analysis — a most difficult task. Absent any standardized set of evaluation tools and models this question could go unanswered for a long time. Yet, it seems reasonable to have a standard set of evaluation models and criteria for so critical an area. Why they have not been forthcoming is not clear and one can only speculate as to the causes.

The past and future

In the modern era we have indeed learned a great deal about these hemoglobin based red cell substitutes. Moreover, what we've learned is useful and practical now and for the future of developments in the field. A variety of solutions with varying composition and characteristics have been tested in clinical situations with some reasonable degree of success. Because the models and experimental designs are different it is difficult to make comparisons in a meaningful way. Success in Phase I clinical testing has been reasonable, for here the models are relatively well proscribed and comparison is possible to some degree. That these solutions report minimal to absent toxicity to the kidneys, liver, lungs, heart and coagulation system is encouraging.

There have been adverse effects reported in many of these trials. The fact that preclinical testing does not always predict the outcome in humans is not new. Nor should it discourage development for each of the adverse events can serve as a stimulus to address another important question of physiology that may be relevant to the progress of a solution or the field as a whole. There is opportunity in the exploration of the causes and mechanisms of adverse events that will provide for future generations of products.

It is important to emphasize the need to really explain the basis for the adverse event and not just provide an intervention that eliminates it or alters the response. The possibility that the observed event is linked to other systems and their underlying physiology, as yet not revealed by the testing, is real and needs to be noted. Key to any of these efforts is, of course, the use of appropriate models with proper controls in well designed experiments that can provide answers to the questions posed. There is a small quiet plea for standardized testing in models well controlled for both positive and negative effects to address this issue. More importantly, it is a most difficult undertaking for the characteristics of the hemoglobin and the solutions pose problems to the design of the experiments, for so many variables lead to a degree of complexity that is not easily addressed. The experiments are complicated by the need to address all of the variables in a systematic manner to uncover the true effects of the agents in question. That is an expensive and unwieldy process to be sure. Nonethe-less, it is essential if the evaluation is to proceed in an orderly and efficient manner. The ability to compare solutions, absent such models, is lost. The ability to compare clinical models for testing becomes limited and near impossible. At some level, this ongoing dilemma must be addressed scientifically by all those concerned if real progress is to be made.

Despite the developments of late and the large body of accumulated knowledge on red cell substitutes, especially those with hemoglobin as a basis, there is a great deal we really do not know. One area of concern deals with the ADME — absorption, distribution, metabolism, excretion — of these modified hemoglobin solutions. That information is crucial to real assessment of toxicity. That these solutions will interfere with routine laboratory testing, artifactually increasing or decreasing a laboratory value by its physical presence or by some effect on the metabolism needs to be addressed. One can reasonably expect the mechanisms that process hemoglobin to be exercised and thus in response liver enzymes will be elevated.

Extrapolation of the micro-physiology studies in animals to the clinical situation has been addressed earlier. It remains an issue not so much because of an absent correlation but because of the absence of a direct correlation in the clinical setting. It would be difficult and foolish to deny there is a relationship for we all know the cell physiology defines and determines the organism's survival. What is absent is the relationship of the observed events and clinical events that require an intervention to assure a better patient outcome.

Predicting the effects of these solutions in the presence of disease is another problem that must be addressed. How will these agents function and clear in the presence of hepatic and or renal dysfunction? Will there be an augmented effect or an attenuated response to these materials? This is especially true of the solutions with vasoactivity as the true basis of this physiologic response is still not clear. Similarly, the gastrointestinal discomfort observed by some patients is an issue for evaluation as the mechanism is not at all clear although the effector, nitric oxide, may be the active agent. How the nitric oxide absorption is triggered and by what mechanism is a far more important question and begs the investigation into underlying causes and not just a proposal to eliminate the symptoms with pharmacological agents.

As the area of application expands will one solution meet all of the perceived needs? Will there of necessity be unique solutions for unique applications? These are real and important issues that need to be addressed for the answers will direct some of the development in the future. Of course, the commercial goal may be some unique application for the return on investment could then be greater. The fact that the solutions now garner any number of new descriptions including oxygen therapeutics tells a tale of the future for the unique properties will need to be tailored to applications and then established as valid treatment for the condition proposed. In some instances this will be a direct challenge to the prevailing common and conventional wisdom of the times and acceptable clinical practice. A challenge of that sort will require a great deal of supporting evidence to achieve full recognition and wide spread use.

Current dilemmas

There are any number of areas where development has been delayed or held up producing the dilemmas faced in getting these products to clinical application. The

well recognized regulatory burden must be noted. The regulatory agencies — everywhere — have a job to do and that is generally to protect the population. How they do that and impose the regulations for safety and efficacy varies from country to country and is no doubt steeped in experiences of the past influencing the decisions of today. Some way to improve the process is needed and some degree of standardization is required. Agreement of the regulatory agencies on models and analysis and definitions of outcome as well as toxicity would be helpful.

The indications for transfusion are changing. Alternative therapy is available as we learn to apply the lessons learned from an analysis of oxygen delivery physiology to patient care. New guidelines and transfusion paradigms have emerged and alternative strategies are available to assist in selecting options to or for transfusion of red cells to minimize the perceived risks [5]. Surgery too has evolved and the modern techniques are associated with less blood loss and when there is expected blood loss the use of cell salvage or hemodilution is appropriate. Clearly less blood is used for routine cases than ever in the recent past. In addition, there is an greater appreciation of resuscitation physiology and with that understanding has come about changes in resuscitation philosophy. Small volume infusion is gaining acceptance and the use of red cells is now limited in the early resuscitation period. The use of red cell substitutes in the early periods may be effective as part of this new approach to volume replacement.

Given the developments it is still likely there is a place for red cell substitutes in the world of surgery as it is now practiced. In a recent analysis fully 60% of the current use of blood in surgery could be replaced with an appropriate substitute with only oxygen carrying ability and some volume expansion capacity [13]. Thus there remains the possibility that the current generation of hemoglobin based red blood cell substitutes will be useful and effective in decreasing the use of allogeneic blood and thus effect some degree of decreased risk for patients. In some context this will also result in transfusion avoidance which is also a perceived desirable objective [2].

Summary comment

Many of the problems of "old" have been resolved and there is great and reasonable progress being made. For those areas external to the process the same constraints may exist and will continue to hamper or impede development. The need for open exchange of ideas and concepts is real. The commercialization of the development and its removal from the academic environment is not beneficial to the scientific inquiry necessary to move these products forward nor is it sufficient to bring on the next generation of products with even more complex effects and composition. There must be some level of standardization to the process that will allow direct comparison accounting for all of the known variables as noted.

The newer problems are the result of different forces and aspects of the development process, likely driven by different entities with different goals. The science and

technology have advanced sufficiently since the early days to make these solutions a clinical reality. Science and technology will continue to contribute to the development and progress of the field. The new problems arise from concepts and ideas that may be afield from the intent of the product. Integrating all that information is part of the new problems that are seen, the dilemmas of the present. It is hoped that these issues and enigmas do not prevent the evolution and application of useful and effective hemoglobin based red cell substitutes in the future.

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E. Tsuchida (Editor)

CHAPTER 9

Round Table Discussion: "The Target and Assessment of Clinical Tests"

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M. Takaori, *Kawasaki Med. College, Okayama, Japan*

Panelists
S.A. Gould, *Northfield Laboratories Inc., Evanston IL, USA*
R.F. Caspari, *Somatogen Inc., Boulder CO, USA*
T.H. Schmitz, *Baxter Healthcare Co., Round Lake IL, USA*
P.E. Keipert, *Alliance Pharmaceutical Co., San Diego, CA, USA*

CHAIRPERSON (TAKAORI): Now we are going to start the round table discussion with each speaker who has made his presentation this morning already. But we would just now make discussion focus on the safety of blood substitutes in practice.

CHAIRPERSON (GREENBURG): We have five different "solutions" represented here. How do we effect the comparison between those solutions so that the audience or the practicing clinician knows which one to use, assuming they go through to acceptance and clinical approval in near future?

GOULD: That's a good question. And I think that each speaker today touched on it to some extent. You've heard many people say that blood substitutes are an inappropriate classification for these products. I think the way to put that into context is to say that blood substitutes may be inappropriate depending on the clinical indication. So, I will start with our own experience.

What I tried to review for you was the initial goal when we began many years ago to develop a blood substitute. Maybe I will be a little provocative and say that I think for the purposes that we have focused our efforts to date, blood substitute is acceptable, where I think you used the term "red cell substitute" in your talk that they are just more accurate. I think "blood substitute" has a certain common usage. Our focus has been this large volume blood replacement, really with the solution that does what blood does, and that in this setting there are some particular benefits in terms of safety and logistic ease of use.

I think that for some of the other indications we have heard about, looking at the benefits of a very vaso-active product by design, that would not be a blood substitute, and I think there are some places in between.

So, how to compare, I think the answer will actually come if all products are approved with the clinicians. I am not sure the point you are trying to get to it at the end of your presentation, but I think many of those answers will occur with those of us that are clinicians here. At least, three of us on this Panel know that no matter what we say, or what the regulatory authority says, it's ultimately the practicing clinicians who are going to determine how and where each of these products should be used.

So, I think that everybody in clarifying their clinical development plans should try and explain what their focus is. And I think you've heard that today; I think you've heard a variety of different uses.

CASPARI: I think, clearly Dr Winslow touched on it today (Chapter 2), the potential need for these products, especially one thinks beyond — just the industrial world is so huge that just from a capacity commercial point of view, we have always felt that there will be a need for different products and several products on the marketplace. So, in that sense, I don't see competing approaches necessarily to reach solutions. We are encouraged by the progress of the field, and feel that progress that is made by any approach has benefits to all. And I do think things will sort themselves out eventually in terms of indication and in differences, and that is very true for any pharmacological area, and pharmaceutical area, that there are several products that may have distinct uses.

I think it's obvious from our point of view we are extremely encouraged by the recombinant approach, in that we feel being able to engineer the molecule, as I tried to touch on briefly today (Chapter 5), and there will be more specific data later on from others, that that ability to engineer the molecule allows us to address some of these potential issues in a very specific way.

CHAIRPERSON (GREENBURG): Dr Schmitz, you have taken the term "red cell substitute" to a different level as Dr. Gould pointed out (Chapter 4). I happen to think it is a more appropriate term for use in the context of this meeting. You have an oxygen carrying therapeutic agent for which you have shown some very nice clinical data. Are all of these therapeutic effects beneficial?

SCHMITZ: I will go back to your original observation. I guess from my point of view, I think that you've got a group of solutions here that fit generally in a class. There are probably differences between those solutions. I think complicating that, like if you look at the pre-clinical data, and even if you look carefully at the clinical protocols, you also have another complicating factor here, which is that, the approach to developing these products is very different. So, you have both potential differences in products and you have potential differences in the protocols that the various companies have elected to run to evaluate products. So, it becomes very difficult to make close comparisons.

And I think the scientist would love to say: Well, why don't you give me one set; give somebody all five products, and I am going to run my protocol. Well, everybody has got "my protocol". And I guess an observation I would make, although we would like, and that might be scientifically intriguing; if you look at studies that have been done on innovation in general. That's exactly what these companies are all involved in. Doing something that nobody has done before, and you look at studies of how organizations accomplish innovation; it turns out that the most successful. Those that are successful are characterized by systems where you have four or five competing approaches to solving the problem.

I guess that if it's taken from a product development, innovation kind of thing, I think, we are following that model, we are perhaps disconcerting scientifically. But I think from the standpoint of "Are we going to have a success of one, two, three, four or five products, we probably have the best system going right now in terms of a lot of very good minds thinking differently about how we are going to get these products into the clinic".

CHAIRPERSON (TAKAORI): Dr Keipert, your product is somewhat different from other products. Do you have any idea to change a clinical indication for your product?

KEIPERT: Well, I guess there are two issues here. One is Dr Greenburg's question about comparing different solutions, and I think the operative word is "different". And they are, in fact, different solutions. We have certainly found ourselves in the position where people tend to view fluorocarbons as all being the same. People have a historical knowledge of Fluosol, the first generation product that was developed by the Green Cross, which is very different from the second generation product, perflubron emulsion, that Alliance has developed more recently. We always struggle with that because there are mindsets based on old Fluosol literature, describing safety issues. The obvious example is something like complement activation that was seen with Fluosol, which is no longer seen with the second generation products.

These products are very different, so making comparisons really is not very helpful. We have done many toxicology studies where we used Fluosol as one of our control arms, because we knew that the regulatory agencies had experience with Fluosol. So if we could show a certain profile, and show that we were at least as good, or certainly not worse, then that would be meaningful. The more we did that, however, the more we realized that it was more important to test our product and to look at how it behaved in our protocols.

CHAIRPERSON (TAKAORI): Now, Dr Gould, I just want to ask you about some adverse effects: When you infuse your product to the patient, as you presented this morning, you can expect a couple of clinically beneficial effects of your product but you did not mention about any adverse effect related to your product. For example, is there any clinical manifestation (symptom or patient's claim) or any side effect on your product?

GOULD: We have not seen any serious adverse effects, or unexpected effects at all that we feel are related to the solution. I gave multiple summary results; the last trial, which was the randomized trial, comparing patients who recieved only blood, with those who received the blood substitute first, up to 6 units of the polyHeme; there were no differences in the number of adverse events.

Now, some of these were clinically injured, trauma patients; so there were events such as infections; you may see some coagulation disturbances; you may see a variety of other events which are common in postoperative trauma patients. Then we feel that comparing group to group was the best way, to be certain that there was nothing that we could attribute in any way to the infusion of the product.

I think it was Dr Schmitz or somebody else said, you can never be certain, but to the interpretation of the investigators as well as our interpretation as we review the data, there is nothing that suggests there is anything that's due to the infusion of the solution itself.

CHAIRPERSON (TAKAORI): Dr Greenburg, you mentioned about some nausea and vomiting after infusion of your product. But is this a very important thing for the clinical application?

CHAIRPERSON (GREENBERG): It may be an important event. I have mentioned abdominal pain, bloating and general abdominal discomfort as well as esophagospasm. In the normal human subjects these symptoms were upsetting. Having seen these effects, the clinical investigation team at Hemosol obtained esophagograms — barium swallow studies — on two of the subjects while the problem was present. There was clear demonstration of esophagospasm with these studies. Intravenous injection of Buscopan — a smooth muscle relaxant eliminated the symptoms within 10 minutes. In a couple of less symptomatic cases oral antacids containing calcium were effective at alleviating the symptoms.

It is one thing to recognize the symptoms, side effects or adverse reactions. It is quite another to appreciate the underlying and mechanism responsible for those symptoms. This area of physiology needs to be pursued. We really have little data on what is likely to happen in large dose studies or in multiple repeat dose studies with respect to the GI symptoms. The basis for these effects needs to be explained and if possible the cause eliminated from concern for the confusion resulting could inhibit the development and application of the entire family of products.

CASPARI: We did the esophageal displeasure in our awake volunteers, in our human subjects. We did some studies both in humans and in animals looking at esophageal motility. We've found that there is an opossum model, an animal model; for some reason, the esophageal physiology of the opossum approaches that of the human quite well. And I think we feel that very much this is related to nitric oxide binding. What was quite interesting, though, is when we moved from our volunteer studies into patients. And now we have patients under anesthesia receiving of the drugs, we did

not observe this phenomenon. Now, you could say perhaps that's timing; that all of these findings, we feel, are very transient. They occur and disappear quite quickly. So it could be that this was just a timing issue. But perhaps it's more an issue of timing and the counter-effect of anesthesia in other drugs.

It's quite interesting that even though we know our product circulates even as much as 36 hours at higher doses, we have not had one complaint from a patient about any kind of esophageal discomfort, or difficulty in swallowing; this was something we only observed in awake volunteers.

SCHMITZ: Our observation has been that I don't believe we have had any esophageal complaints.

CHAIRPERSON: Any gastrointestinal complication?

SCHMITZ: Not severe. Few nausea, or vomiting.

CHAIRPERSON (GREENBURG): I think that it's interesting to focus on that particular area, and I will make some comments.

After probably eliminating half of the opossum population in up-state New York last winter doing the same model, we came to very much the same form of conclusion that there is an interesting physiology there that we can make go away fairly easily. And the mechanism of it going away in the clinical situation in the patient can be explained by the fact that every general anaesthetic is a vasodilator, No. 1, that's how they work. No. 2: At least in continental North America, the United States and much of Canada, the mixture of drugs that anesthesia uses before a patient is put to sleep will essentially block every smooth muscle reaction there is. So there is no way of telling what's going to happen. And it goes away. And in our few Phase II patients, it has not been an issue yet, and we are at the slightly higher doses.

One of the issues, which came up, and I find it absolutely fascinating, is what is a unit? And we are still talking about things as units of blood when in modern therapeutics we talk about milligrams and efficacy, et cetera. Dr Gould, you want to handle that one?

GOULD: I am glad you brought that up. I was going to come back to it, as I had another thought about how to compare products. My concept; historically, this began because of the comparison to a unit of blood. And I was not around when that started. I know you (Prof. Greenburg) were probably there when blood started.

CHAIRPERSON (GREENBURG): I was.

GOULD: If a unit is 500 ml, and we say functionally it delivers about 50 grams of hemoglobin based on the loss of the red cells during storage in the average storage time. You and I are surgeons who know that it's very hard to change the physician behavior. I think surgical behavior is probably the hardest to change.

The notion of giving a unit of an oxygen carrying solution has led to an under-

standing that when you do that, you raise the hemoglobin 1 g. Most surgeons don't think of how many grams are in the bag, or they know what the effect is.

So, Dr Moss, G and I, very deliberately with our colleagues focused on having a "unit" that would be equivalent, and I think Dr Winslow had that on his slides (Chapter 2), something for ease of use, not to have to deal with changing physician behavior. So, for us, as I specified the unit, it was important that would be 50 g. There is nothing magic about 50 versus 25, or versus 30 g.

Coming back to your first question about comparing products, each member of the Panel addressed today in some way the indications for their product and the parameters that might be improved. It went from hemoglobin, as I used, to venous PO-2, to blood pressure, and each of us did that. I think that is potentially a problem when physicians have to compare new products. If they have to retrain themselves and learn new practice patterns, I see that as a potential difficulty.

So, for us, there is a very specific reason why we made the unit that way. Now, I will tell you on the upside, I briefly said that, as we have gone to the 10 unit dose or 500 g or 5,000 ml, frankly there have been some very impressive uses at that dose in these massive trauma patients. And the surgical investigators that we are working with have asked us for a larger size at the very least. We are now planning a one 1 l bag. I don't know if we call it a double unit or what terminology we will use. I got a serious request, literally from a very distinguished surgeon, to make a 5 l bag. So I think the concept of unit depends on uses.

CHAIRPERSON (GREENBURG): I don't disagree with that and raise the issue because Dr Caspari talks about 25 g unit, which is in the context of his description. Peter talks about bags of oxygen carrying staff. Baxter has 10% solution. Is it right, Dr Schmitz?

SCHMITZ: Yes, it will be a 25 g unit.

CHAIRPERSON (GREENBURG): But Hemolink's product is 50 g unit. There are some differences. Surgeon who asks for the 5 l, I am pretty sure I know who always wants more. Just the, all over the place, I will tell, I will mention to the audience that there is some evidence of papers ASS hope showing that hemoglobin solution protects warm ischemia. Warm ischemia that we clamp the renal artery in operating room, and leave the kidney without blood flow for one hour, in animal model, and then we reperfused to take up the other side works fine. It works that's something, that probably the most strenuous test there is for the ability of these materials to keep organ alive in the model.

GOULD: Let me complete the thoughts before we distract all. Here we are in operating room, and I am focusing on our approach again which is really replacement of a significant hemorrhage. And surgeon or the anesthesiologist, in this case just, are giving a unit and not sitting in an appropriate increasing in Hb, that something that can meet to inadequate physicians' acceptance script.

I think that surgeons and anesthesiologists when they're giving something to replace lost blood, want to be confident that have conference that when they give a unit they are going to have an appropriate increase in the oxygen carrying capacity.

CHAIRPERSON (GREENBURG): But the problem is most of our colleagues have no idea what an appropriate increase in oxygen carrying capacity is, how to measure it or how to use the data to achieve that sort of clinically useful endpoint. This area of oxygen delivery physiology is evolving rapidly and doses appropriate to some patients may not be applicable to all.

An increase in oxygen carrying capacity needed to reach a given level of oxygen delivery in the specific patient is what is needed. We must get away from "units" or cells and begin to speak of the underlying physiology. Various factors enter into the decision and one critical one is the presence or absence of coronary or other cardiac disease. There are times when increasing oxygen delivery requires increasing carrying capacity and other times when the increase can be effected by improving cardiac dynamics. The components of the system are integrated but can be effected by improving cardiac dynamics. The components of the system are integrated but can and should be manipulated independently to acheve the desirable clinical outcome.

GOULD: Right, I agree.

CASPARI: My think someone for me perhaps, it's, you know, one end of elephant you looking at here, and I do understand there is carnation the notion of one is surgeon is used to in the whole concept of unit, but I also I think I understand is pharmaceutical developer. And someone who's been belonged in putting several drugs on the market, and that the other way is to potentially to look at this. I think that out feeling is that it is one thing we pretty certain about is their importing ability of that molecule end up with to deliver oxygen very efficiently. And then for we are talking about in oxygen tissue delivering therapeutic, and in that sense the reason we chose 25 g was there that we believe that 25 g is a very equal. In sense of oxygen delivering ability to one unit of attacked red cell, and it's really that therapeutic ability, that is key here. While I don't disagree that the time can be hard and big challenge to take on current practice, I can also tell you that it can be a very exciting challenge for pharmaceutical development, point of view. And one I actually look forward to, I think that will be great story to tell, and I come to believe that while I think the red cell was designed very cleverly, and it is a very good reason why Hb is in a red cell. And then under the acute circumstances actually having outside of red cell and that with ability to deliver oxygen very potently could be beneficial in effect. And so then I would like to educate physicians much more about the therapeutic implication not happen think so much of a unit, but happen think about oxygen capacity. And when we think beyond the operating room, beyond the traditional blood substitute, potentially even dealing with an acute hypoxia in the broader sense. Some of ischemic complications and then I'm not sure that thinking of unit is really beneficial.

CHAIRPERSON (GREENBURG): That is an excellent comment and reflects some of what Dr Winslow said about the ever growing and evolving list of indications for red cell substitutes. These materials have properties different from blood and as such may have more favorable effects. Their unique properties can be used to directed ends once they are understood in the context of the bigger picture. I think Dr Caspari's group has shown quite well that there is benefit to having less hemoglobin under certain circumstances, independent of the units used. This has been shown to be true in both animal and clinical models with at least two solutions to date. You are addressing the right issue.

SCHMITZ: I reinforce what Dr Casperi has said. And we do believe that we will not require one or more replacement in most situations. Unfortunately I also believe that will be a harder road to follow. I believe we will have to provide information to clinicians on how to properly use the product. I think it's inevitable because the product does not to behave as a gram to gram replacement. It behaves differently so that we're going to have to face up to that challenge.

CHAIRPERSON (GREENBURG): To should be having a session "adoption on innovation", you and I should have this individual discussion of how we get physicians to change their behavior. They actually read very little literature in that area. It will be wonderful experiment. I have to consider in next carrier.

KEIPERT: I would like to add some comments regarding unit dosing. We think of blood transfusion one unit at a time. There are no dose limitations so we keep giving it until you achieve your desired effect. These oxygen carrier drugs clearly have some degree of dose limitation. Alliance has a fluorocarbon-based product, so we tend to think of it more as a drug because of we've always dosed it on a gram per kilo basis.

If we think about that for a moment, we must consider whether we should be dosing on a unit volume basis, i.e. give everybody 500 cc or giving everybody 1 or 2 g/kg. There are huge ranges in body size in patients. We dose patient which as little as 60 g for low body weight of lower doses to see efficacy, but we go up to doses over 250 g in large patients. So when we compare blood half-life, oxygen carrying capacity between different patients, all of these things are dose-dependant. But if we go to unit-dosing concept, then we are going to get more benefit in small body mass, 50–60 kg woman, as opposed to the 80, 90, or 100 kg male. So these are all issues that really complicate the whole story of how we dose these products, and how to compare "units" between different products.

CHAIRPERSON (TAKAORI): Is there any question or comment on this subject?

BAKKER: I have seen in treated in Europe observation of Hemoglobin urine. My question is which size of the molecule with that low-dose will be excreted in urine of non-crosslinked hemoglobin molecules. And did you ever observed only patients, which had some problem with that kidney.

SCHMITZ: We did not observe any kidney toxicity, using a 64,500 molecular weight crosslinked material.

BAKKER: We even causing that went through, the otherwise did cause no signs on the kidney toxicity. What doses the amount compared to the amount of hemoglobin.

SCHMITZ: Very very small. Red change in urine?

BAKKER: And from the regulatory point of view, what would be acceptable level of uncrosslinked hemoglobin contamination that is allowable. From my kidney work, we guessed that we can go up to 2% of non-crosslinked hemoglobin.

SCHMITZ: We are much below that. Less than 0.5%.

BAKKER: Because we linked up higher than 2 or 3%. We apt to shock could show kidney toxicity into rat model?

CHAIRPERSON (TAKAORI): Is there any question and comment?

WINSLOW: I have a question for Dr Gould. About 10 years ago, Northfield was the first company to start clinical trials. There was a patient who had some side effects, but I don't really know details. Ten years later, in retrospect, do you have any new thoughts on this case?

GOULD: We've been at it so long. In the first group of volunteers; we had couple of reactions. The patients had a sense of anxiety; they describe subjectively respiratory difficulties. I think twice or more when we measured the PO-2, it was minimally reduced, and in volunteer it was not. And in retrospect, it looked to us like an allergic reaction, almost — this was really at the beginning, and just to set the stage at the time, we were the first to initiate human trials. We had some independent observers there, and two critical care physicians, who felt they just had a transfusion reaction; that's what it looked like.

As we sorted that out over a six-month period, it appeared to us that we had not achieved the level of stroma reduction that we have now.

Those are the major changes that we have made during that transition, and we have never seen any reactions since. So, for purposes of describing our data, we usually include those early recipients since the traditional toxicities that occur with hemoglobin solutions; never occurred with those people.

FRATANTONI: I have a comment and a question. The comment refers to what I am going to mention tomorrow morning. And since I am speaking at 8:30 and in competition with the session on microcirculation, people may miss this.

Referring to the question of comparing products, what I am going to mention is that when the FDA made this proposal on efficacy early in 1994, the comment was

made then that it would be good for manufacturers to do clinical physiological studies, comparing physiological effects with clinical observations, and that perhaps piece by piece we would begin learning more about how these products work, and that hopefully by seeing these data, and seeing them published, we'd all learn more.

I think it's fair to say we were disappointed; there was not an enormous amount of material published. There were a fair number of studies done.

And so, a comment I am making is a speculation: Would it have been better for the field, in general, had the FDA mandated that all products be tested in a series of standard models? Can we answer that question now? It's a speculation. But again, the question is would we all have been better for that? Would it actually speed up the road to approval, because observers would have a better idea of how these various products work, and perhaps feel more comfortable in approving one or more of them? That's my comment.

A question I have is to Dr Caspari. Your product, your recombinant product, the expression system is *E. coli*; you didn't mention anything about problems with endotoxin. Could you say something about that for us?

CASPARI: I'm not sure what problems you are referring to. You mean endotoxin levels within the product?

FRATANTONI: How successful are you in getting rid of endotoxin, the historical problem of difficulty in removing endotoxin from hemoglobin, the data on levels, and so forth.

CASPARI: I think we have been extremely successful in removing endotoxin. In fact, we are very consistent in current clinical batch material of having endotoxin levels that remain below the level of detection with the most sensitive assays, and further the assay development has progressed, as I understand it, over the years.

I am not the expert in process and manufacturing, but what my colleagues tell me is that, as we make, talking very simplistically, now make the product in *E. coli* lysed cells, and then take this through a chromatography step that captures a lot of the unnecessary and unwanted material, we reached 99.9% percent purity at that point, then go through a series of ultra-filtration steps. And endotoxin has really not been a problem.

I think, also, to emphasize that, on the clinical side, it's interesting that very early on in our development, we saw these kinds of flu-like symptoms and fever complex type symptoms. And as we made improvements in our manufacturing process, those have gone away. We do not see this any more.

We have been fortunate in studying our product both in acute, high doses, but in our hematopoietic program, repeat dosing at lower levels, but those lower levels in some patients reached 3 g, three times a week for eight weeks. And again we see nothing that speaks of endotoxin or immunological problems. So, we feel we have been quite successful; it's not a problem.

One of the presentations here best speaks to what was theoretically in the

literature, this potential issue of interaction between endotoxin and hemoglobin. And we do have some data in animal models that, at least in our hand, show that it is not a problem. So, we believe the endotoxin issue is very much behind us.

SEKIGUCHI: I am Sekiguchi from Hokkaido Red Cross Blood Center. And I would like to ask another point.

Recently, people involved in a blood program are very anxious about the risk of Creutzfeldt-Jacob disease due to abnormal prion and prion transmission. Even we use human blood for the source of hemoglobin; we cannot eliminate such risk of prion transmission.

So, do you have any idea to eliminate such a risk of Creutzfeldt-Jacob disease and prion transmission?

CHAIRPERSON (GREENBURG): Anyone want to — Well, Dr Keipert, it's not an issue.

KEIPERT: I will pass. It's not an issue for perfluorocarbon emulsions.

SCHMITZ: I think it's an issue for us. And I guess since there are no tests and at least no direct test links at this point in time. What Baxter is doing is monitoring the progress in this field. This is a corporate issue, basically, for our Company. We not only are involved in blood substitutes; we are involved in plasma proteins. And we have a blue ribbon panel of virologists and experts in this area to keep us advised of what's going on, and whatever is possible to do to address that issue, it will be done and it has to be done.

But right now, there are as many theories and possibilities, donor restrictions, and all the other things that might be done. But it's always going to be disturbing. I think that would be the one thing I would comment on Dr Winslow's presentation. Yes, mainly blood is safe for what we are testing. But I think that's part of the perception problem we have with the patients. They say: "Well, what don't you know?" Because it wasn't too many years ago that there were some real nasty things we didn't know, and had things happen.

So, all we can do is keep abreast of the various developments, and hopefully contribute to understanding with the research that we do.

WINSLOW: I want to follow up on one thing Dr Caspari was talking about, the erythropoiesis application. To me this is still a little bit counter-intuitive, because I would have thought that hemoglobin is a good oxygen carrier, would depress erythropoiesis and not stimulate it.

And so, isn't the most logical explanation for increased erythropoiesis to decrease oxygen supply to the kidney rather than some other effect, and do you have an explanation for why erythropoiesis seems to go up after your product is administered?

CASPARI: Excuse me. No, I don't, but I think our hypotheses are not around oxygen as much as I am speaking in very generally and rheologically here, it at least makes sense to me that under conditions of hemolysis, where you are dumping free

hemoglobin into the system that there is some kind of signaling mechanism back to the marrow to make more red cells. The amount of hemoglobin that we use, the dose that we use, we believe, is not related to oxygen, but it may have something to do with heme; may have something to do with iron. It's not clear what it is.

But there is some evidence in the literature, and with some preliminary animal data that we have, that hemoglobin, in fact, may stimulate hematopoiesis, and not only the red cell line but potentially the platelet line as well. Whether this will hold up in patient studies, I don't know. We are completing those studies; will be in a data analysis phase soon; so, you know, we will see.

But I don't know what mechanism is —

CHAIRPERSON (GREENBURG): I guess "hypoxy" wasn't the right word, but have you looked, for instance, at real blood flow to see if there is a reduced renal blood flow, as opposed to a hypothesis?

CASPARI: No, we have not done that.

GOULD: You know, I think, when Dr Winslow said there is a historical precedent for that, the Ambersen papers actually talk about that. Let's go way back; we have seen people focus on the iron, or whether there is some nutrient effect actually on the marrow.

But I will take the opportunity to ask Dr Winslow. Are you using erythropoietin on your end stage renal diseases? Is this being done with or without erythropoietin?

CASPARI: We would have loved to have done it without. We couldn't find a patient population, at least within reasonable reach in kind of modern societies, of end stage renal disease patients who were not on erythropoietin. That's become standard of care. So, the study we have done has been with the erythropoietin.

In fact, I think the contribution we may end up making, if we do make one, may have nothing to do with the product. I hope there is something to do with the product, but at least I think we have been able to struggle through and teach nephrologists how to properly dose patients with the iron and erythropoietin. Because as part of that study we have to stabilize patients in terms of their iron dosing and their erythropoietin dosing, and that took a lot of the effort. And so, what we have tried to do is look for a synergistic or additional benefit on top of the erythropoietin.

Now, that design could be such that those patients are maximally stimulated, and we don't see any effect, which is one reason we also studied another population who do not get erythropoietin, and that was the mild dysplastic population.

So, yeah, all of these patients are on erythropoietin.

CHAIRPERSON (TAKAORI): Dr Greenburg, any comment on this?

CHAIRPERSON (GREENBURG): Two quick comments about this.

One, in the Hemosol Phase I trial in normal human volunteers, we actually measured plasma erythropoietin level at different times and the process. And we did not see any change in erythropoiesis or in reticulocytosis. But those were normal iron loaded patients, and we might not have expected to see any problem in that group.

Another division of Hemosol, the stem cell group, has shown a remarkable synergy and effect between hemoglobin and erythropoietin in the stem cell models, which have been published, showing a tremendous increase in efficacy of erythropoietin even in the presence of hemoglobin, because the stem cell appears to be able to use the porphyrine iron and not break it down. So there is some real possible hope there.

Even though if you recall the African studies of Feola, and some of the early studies, those patients all had an erythrocytosis after they had hemoglobin. I think even Savitzki's patients did. So, there are some interesting elements there which we don't quite understand, and perhaps they are opening up a whole new area.

KEIPERT: I have more of a comment than a question regarding Professor Sekiguchi's question on CJD disease and prion removal.

I don't believe there is anybody here from the Biopure Corporation, but at past meetings Dr Jacobs has presented in public that Biopure claims to have a validated method for a prion removal, and I was just wondering if anybody knows anything about that, or if there is any investigator that has worked with the Biopure product who could maybe tell us more about this.

FRATANTONI: This was mentioned in public. I don't think it's a method for removal. I think what's been done is to look at the infectivity of material that has been spiked with a scrapie strain, and then look at that infectivity after the material was processed, so that what they showed was the processes, including the purification methods, and the polymerization chemistry, had some effectiveness in decreasing the infectivity of the scrapie strain.

This same sort of technique has been used by the plasma people in trying to determine whether or not the very much processing of plasma into plasma derivatives can decrease the infectivity of scrapie used as a model.

So, there is a possibility that those products we are talking about here, that are produced from red cells, that the very much process of manufacture could decrease the infectivity. Of course, the ability of Creutzfeldt-Jacob disease to be transmitted by blood has been classified as a theoretical possibility. As experiments are being done, I am hearing that that theory is getting to look a little more real than it did a year or so ago.

CHAIRPERSON (TAKAORI): Well, we may still have lots of question or comment on the subjects, but we would like to close this session because it will make Dr Nishide happy. So, anyway, we are very glad, this session was cooperated nicely with all of participant and audience.

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 10

Biophysical Criteria for Microcirculatory Efficacy of Blood Substitutes

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Introduction

Historically blood losses leading to hemorrhagic shock were treated by intravenous fluid infusion with the aim of restoring systolic blood pressure and urine output, and stabilizing heart rate. This goal has evolved to the present focus of bringing tissue oxygen delivery to near normal levels, as expressed by arterio/venous oxygen pressures and blood oxygen content. The original end points for evaluation of the efficacy of blood volume normalization were systemic parameters and could only provide information on the cellular events on a global basis, a scenario that persists with present criteria for efficacy in blood volume restoration. This is primarily due to the fact that the nature of oxygen distribution in the organism, and its control have not yet been fully established, while new methodologies for the study of microvascular events are yielding data that challenge established concepts mostly based on theoretical foundations.

The ideal fluid to replace blood in an emergency is an artificial fluid with identical physical and biological properties, a goal of difficult realization due to complex nature of blood and still imperfect knowledge that we have of its properties and functions, particularly regarding tissue oxygenation. Thus if tissue oxygenation is to be regarded as the critical factor in evaluating efficacy, it is necessary to understand this process at the level in which it occurs, namely the microcirculation. It follows that the design of a plasma expander that works in synergy with blood requires the simultaneous solution of biological and transport problems operational at the level of microscopic blood vessels, where oxygen is unloaded through the contact between blood and tissue, in a processes where diffusion and convection become superposed.

Present status of tissue oxygenation data

Oxygen delivery by the microcirculation is to the present dominated by the so called Krogh cylinder formulation [1] that models oxygen transport from blood to tissue by

single capillaries, tissue oxygenation being summation of the behavior of the unitary components. This analysis sets capillaries as the suppliers of oxygen to the tissue, a functional mirror image from events in the lung, where they were first observed.

The introduction of microtechniques for measuring pO_2 in the tissues has shown that significant amounts of oxygen exit the arteriolar network, challenging the role of capillaries in tissue oxygenation, and recent studies of Intaglietta et al., 1996, [2] demonstrate that in fact most of the oxygen, at least in resting skeletal muscle and mesentery is delivered by the arteriolar network. The presence of the substantial longitudinal oxygen exit [3] as blood transits the microscopic vessels prior to arriving to the capillaries also requires the existence of significant diffusional fluxes out of these vessels. These fluxes could not be satisfactorily explained in the past because the technology was not available to measure the necessary tissue pO_2 gradients. Current understanding of the biology of microvessels indicates that their vessel walls are important sites of biological activity which should be driven by a corresponding level of metabolism, which would result in the presence of large oxygen gradients within the arteriolar walls. These large oxygen gradients may in principle be the consequence of a large diffusional resistance within the wall [4], or to the enhanced metabolic rate. These divergent explanations lead respectively to a small or large rate of oxygen exit from the blood vessels, the difference being about an order of magnitude (factor of 10) [5]. Mass balance considerations, however, indicate that the rate of oxygen exit is large and commensurate with the existence of a large oxygen sink within the vessel wall.

The new technology of phosphorescence quenching [6,7] has been critical in elucidating many of these issues because it has for the first time allowed to measure both intra and extravascular pO_2 with the same methodology and therefore in the same experimental setting. This technology has provided data that allows for the in vivo analysis of critical assumptions to the Krogh model and its consequences for tissue oxygenation. Results for skeletal muscle at rest and subcutaneous connective tissue show that:

1. Capillary blood pO_2 is only slightly higher (about 5 mmHg) than tissue pO_2.
2. Arterio/venous capillary pO_2 differences are very small, because tissue pO_2 is essentially uniform.
3. The only tissue domain where pO_2 exhibits large gradients is the immediate vicinity of the microvessels, a tissue compartment whose main constituent is the microvascular wall.
4. The major portion of blood oxygen is delivered to the tissue by the arterioles.
5. Under basal conditions tissue capillaries only partially serve to supply oxygen to the tissue. They may be a structure to expose the endothelium to blood in order to fulfill the large oxygen demand of these cells and insure the removal of metabolites consequent to tissue respiration.

Some of these findings have been previously reported, but have laid dormant because they could not be readily verified with the microelectrode technology. It was

known as early as the 70s that the capillaries are not the only source of O_2 in the microcirculation by Duling and Berne, 1970, [8] since significant O_2 delivery was measured from the arterioles. The technique, however, based on polarographic microelectrode measurements, did not allow for consistent measurements of microvascular blood pO_2 measurements.

These findings suggest that oxygen respiration in some tissues at rest (skeletal muscle, connective tissue) is a prioritized process, that satisfies first the oxygen demand of the endothelium (and smooth muscle) and secondly that of the parenchyma. A corollary to this hypothesis is that the microcirculation is designed for a specific distribution of oxygen tension that is in part determined by the shape of the oxygen dissociation curve for hemoglobin and placement of blood with specific oxygen tensions at specific microvascular locations as shown by Tsai et al., 1996 [9]. Thus tissue oxygenation is not only driven by the pO_2 gradient between blood and tissue, but also results from the interaction between flow related phenomena, blood oxygen carrying capacity, and diffusion. This physical triumvirate of processes is also directly coupled to active mechanisms that sense oxygen partial pressure in both tissue and blood, and accordingly partitions oxygen delivery between the arteriolar and capillary circulation.

Although tissues vary in anatomical and physical placement of the location of the fulcrum that separates arteriolar and capillary oxygen delivery, there is evidence for the existence of an adrenergic sensor system that partitions arteriolar from capillary oxygen delivery [10]. The knee of the oxygen dissociation curve for hemoglobin is located at the same level of the arteriolar network as this adrenergic sensor system. Arterioles prior to the fulcrum deliver most of the oxygen in tissues at rest, even when functional capillary density is normal. Capillary oxygen delivery becomes the predominant mode of oxygen transport in working tissue. The shift from passive to active oxygenation, namely arteriolar to capillary oxygenation is mediated by central and local mechanism that modulate flow to increase wall shear stress, leading to a positive feedback process through the release of shear stress dependant vasodilators that insures maximal capillary oxygen delivery, further augmented by decreased diffusional oxygen losses due to higher arteriolar flow velocities and increased functional capillary density. Partition between capillary and arteriolar oxygen supply is the key process of oxygenation control where physical and biological mechanisms interact in a fundamental way in setting tissue oxygenation.

Design of fluid for blood replacement

The problem of restoring circulatory function in the presence of blood losses requires consideration of many interacting biophysical phenomena, where the preliminary task is the identification of the determinant parameters. This preliminary selection is aided by the development of a model that circumscribes the analysis of tissue oxygenation to the interaction of the blood transport properties and endothelium

metabolism, determined by the viscosity of blood, the oxygen consumption associated with the production of metabolic shear stress derived vascular mediators (EDRF), and the blood oxygen carrying capacity and availability.

In circumscribing the analysis of tissue oxygenation as a function of mostly mechanical factors determined by the physical properties of blood, and particularly viscosity of the circulating fluid, we can establish a gage with which to explore the effects of varying the composition of blood with a comparatively large number of oxygen carrying and non-carrying plasma expanders. This approach also allows to identify their relative merits as blood volume restoration/replacement fluids.

The introduction of oxygen carrying and/or non-carrying plasma expanders into the circulation changes the distribution of transport parameters of the microcirculation. In the normal organism these changes engage mechanisms that strive to maintain homeostasis, namely a "normal" distribution of hydraulic pressure, oxygen content and pO_2, and viscosity. These responses in some cases bring the system back to its operating set point maintaining tissue oxygenation normal, however this cannot be assumed always to obtain and tissue oxygenation may be impaired.

In this context the hydraulic pressure imparted to blood is dissipated in the microcirculation. Oxygen gathered in the lung is delivered in the microcirculation. As a consequence, in the microcirculation, there is a hierarchy of blood vessels and transport parameters, larger arterioles being exposed to high blood pressure and high pO_2, while smaller vessels are progressively exposed to lower pO_2 and pressure. The partition of hydraulic pressure and oxygen is a function of blood viscosity. If central blood pressure is regulated, significant changes of blood viscosity may cause major changes in the distribution of blood pressure.

Blood viscosity is also distributed in the microcirculation, because hematocrit varies continuously from the systemic value to less than 50% of the systemic value, as blood transits from the larger arterioles to the capillaries as a consequence of the Fahraeus-Lindquist effect. This is a major effect because of the non-linear relationship between blood viscosity and hematocrit. In larger arterioles blood viscosity is about 3.5–4.0 cP, while in the smaller arterioles viscosity falls to about that of plasma. This has large effects on shear stress and shear stress dependant release of EDRF (NO and prostaglandins) since the circulation is designed to maintain shear stress constant.

In other words, shear stress is also present in a prescribed way in the microcirculation when the system is perfused by normal blood. A molecular oxygen carrying plasma expander is not subjected to the Fahraeus-Lindquist effect, causing the whole microcirculation to be exposed to a uniform viscosity, significantly changing shear stress distribution. These same considerations apply for oxygen carrying capacity distribution, since hematocrit decreases progressively as blood transits in the microcirculation, but it becomes uniform with molecular oxygen carrying plasma expanders. A formula for the relationship between the variables that determine oxygen distribution in the microvascular network is based on mass balance considerations for a segment of a microscopic blood vessel [11,12].

Solution of the diffusion equation in a consuming medium for the layer of tissue adjacent to the blood tissue interface yields the rate of oxygen exit per unit vessel length and the summation of each of the oxygen losses along the vascular network allows to obtain an expression K_n that gives the total loss of oxygen prior to arrival to the capillaries expressed in mmHg, as follows:

$$K_n = \frac{128\mu}{F(Htc)} \sqrt{\frac{2g_o\alpha D}{2}} \sum_{1}^{n} \frac{L_i^2}{n_i d_i^3 \Delta P_i}$$

where μ is blood viscosity; $F(Htc)$: oxygen carrying capacity of blood; g_o metabolism of the vessel wall; α: solubility of oxygen in tissue; D: the diffusion constant of oxygen in the tissue; L_i: the length of each vessel segment; n_i: the slope of the oxygen dissociation curve for hemoglobin in the segment; d_i: the diameter of the vascular segment; and, ΔP_i: the pressure drop along the segment. The derived equation shows two distinct groups of terms. One group is common to all vessel segments and includes blood viscosity, hematocrit or blood oxygen carrying capacity and vessel wall metabolism. The second group is a summation where each term is specific to each vascular segment.

This expression shows the functional relationship between transport parameters that determine capillary blood pO_2 for given changes in the physical properties of blood, furthermore, solely on the basis of the transport phenomena, it shows that better oxygenation results from lowering viscosity. g_o represents the vessel wall metabolism, which had not been previously recognized as a significant component in the management of oxygen distribution. g_o has a steady state value representative of the baseline activity necessary for the living processes of the tissue in the microvessel wall that is directly affected by the composition of blood and flow velocity. Furthermore alteration of metabolism due to inflammation or increased tone should increase in tissue metabolism and therefore lower tissue oxygenation (since the increase in K_n lowers capillary pO_2).

Design applications

When the derived expression for oxygen exit from blood is used to predict the outcome of changing blood physical properties in the microcirculation we find that many of the expected benefits are not obtained. As an example, hemodilution with a colloid such as dextran should yield the same drop in oxygen tension in the circulation [13] as normal blood, and therefore normal tissue oxygenation, a result that is observed up to red blood cell losses of up to 60% [14].

When hemodilution is carried out with an oxygen carrying plasma expander based on cell free hemoglobin, viscosity decreases, but the oxygen carrying capacity of the blood/expander mixture is maintained; therefore, more oxygen arrives to the microcirculation. Paradoxically, excess oxygen engages autoregulation, causing

vasoconstriction, which as shown in the derived expression has a fundamental effect on oxygenation due to the exponential relationship to diameter.

Superposed to oxygen dependent autoregulation is also flow dependent regulation, where lower shear stress resulting from decreased viscosity which is not compensated by a proportional increase in flow velocity, diminishing the production of endothelium-derived vasodilators and further potentiating the vasoconstrictor stimulus.

These effects are found in experimental animals models subjected to blood exchanges with hemoglobin-based blood substitutes when the exchange is carried out to a level that is beyond the self-regulating capacity of the circulation for compensating for variability in the factors given by our equation.

The effects of blood viscosity

It is generally assumed that lowering blood viscosity is beneficial as shown by many studies of hemodilution. However the specific physiological effect resulting from lower viscosity is still not well established. A means for calculating the vascular effect due to lower viscosity is provided by rewriting Poiseuille's equation and expressing a vascular diameter D for the circulation in the following format:

$$D = \left[\frac{Q\mu}{\Delta P}\right]^{1/4}$$

where Q is blood flow rate, μ is blood viscosity and ΔP the arterio/venous blood pressure difference. Utilizing the data from the study of Tsai et al., 1995 [13] where isovolemic hemodilution was compared with isovolemic blood substitution with $\alpha\alpha$-Hb, at 15g/dl concentration and applying the equation for vascular diameter we obtain the result shown in Table 1.

This analysis shows that blood substitution with both a colloid and a hemoglobin solution cause some level of vessel wall narrowing, a phenomenon that is even more noticeable if we consider that stroma-free hemoglobin is frequently associated with elevation of blood pressure.

Table 1

Comparison of changes of vascular diameter for isovolemic hemodilution with 6% Dextran 70,000 Mw. and a solution of 15% $\alpha\alpha$-Hemoglobin.

	Flow rate Q	Viscosity μ, cP	Vascular diameter D
Normal blood	1.0	4.0	1.4
75% Exchange, dextran 70	2.0	1.0	1.2
75% Exchange, $\alpha\alpha$-Hb	0.8	1.0	0.9

Data from Tsai et al., 1995 [13].

The realization that at high levels of blood substitution there is vasoconstriction is important because this phenomenon is associated with decreased levels of functional capillary density, which is a critical parameter in insuring tissue survival during hemorrhagic shock [15,16]. This finding leads to design of an experimental procedure to test whether functional capillary density is related to plasma viscosity during extreme hemodilution, i.e. reductions of the red blood cell mass of about 75%, with the result that restoration of blood viscosity by a 6% solution of dextran 500,000 Mw, viscosity 6.5 cP, to a level of about 2.5 cP for the circulating blood leads to a significant recovery of functional capillary density (about 85% of normal) from about 40–50% at which level animals do not tolerate severely reduced oxygen capacity.

Pilot studies further validate these hypothesis. Artificially increasing blood viscosity during hemodilution with various hemoglobin solutions by adding polyvinylpyrrolodine (PVP, 750,000 Mw) to the replacement solutions show that microvascular perfusion and tissue pO_2 was invariably increased with the more viscous solutions. Similar results have been reported for cerebral blood changes after hemodilution with polymerized bovine hemoglobin mixed with 2% PVP [17].

The physiological effects related to viscosity are directly linked to flow, whereby the endothelium directly senses blood flow via the shear stress induced over the cell membrane by the moving blood. The mechanism relates blood viscosity, μ, and shear rate dv/dr which determine shear stress τ_{wall} at the blood vessel wall ($\tau_{wall} \approx \mu dv/dr$). Production of the vasodilator prostacyclin and NO by the endothelium as well as endothelin [17–20], is a direct function of the shear stress generated by blood at the vascular wall. Consequently lowered blood viscosity is beneficial only if blood flow velocity increases in proportion so that τ_{wall} remains constant, otherwise if flow velocity does not increase to the required level, the net effect of lowered viscosity in terms of autacoid regulation is vasoconstriction and the consequent reduction of functional capillary density.

In view of these findings, it is not surprising that some hemoglobin solutions cause hypertension as a side effect and do not reach their full potential oxygen delivery capacity [21,22]. This reaction may in part be due to scavenging of the endothelial-derived vasodilator NO by hemoglobin; however NO production by the endothelium in order to be effective should be directly transmitted to the underlying smooth muscle, with little possibility for being captured by hemoglobin molecule in solution in blood.

Summary

The development of an analytical model for the transport properties of blood of varying compositions allows to identify the critical parameters that determine oxygen transport to the tissue when blood physical properties are altered. Blood or plasma viscosity appears to be a key determinant of tissue oxygenation because it affects

blood vessels in terms of vasoconstrictor/vasodilator effects that ultimately modulate functional capillary density. Many of the changes in the composition of blood following the introduction of oxygen carrying materials vary its properties within the autoregulatory capacity of the circulation, however, blood viscosity is a non-linear function of hematocrit, and the reduction of the red blood cell mass to 50% significantly changes blood viscosity, to a level that may not be compensated by the circulation. These studies lead to the conclusion that even in normal conditions the increase in blood oxygen carrying capacity does not lead to increased tissue pO_2, unless several parameters inherent to our analytical results are changed simultaneously.

Acknowledgments

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 11

Experimental and Mathematical Simulation of Oxygen Transport by Hemoglobin-based Blood Substitute

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Introduction

In vitro experimental and mathematical methods for simulation of oxygen transport to and from tissue have been of interest for many years. The results of such simulations can contribute to the understanding of processes of basic physiological interest as well as understanding disorders related to oxygen transport such as shock, anemia, and sickle cell disease. Reliable simulation methods can be of particular value in the development and evaluation of alternative oxygen carriers — such as the hemoglobin-based oxygen carriers which are the focus of this work.

In vitro experimental simulation methods can serve as a useful adjunct to direct in vivo measurements. In vivo measurements of local oxygen fluxes and local oxygen concentrations at the microvascular level are subject to well-know difficulties associated in part with uncontrollable variation in both space and time of the measured quantities. The difficulties have yielded several anomalies which have been discussed elsewhere [1–3]. These difficulties gave impetus to the development of the well-defined, controlled, in vitro system used in this work.

Mathematical simulation methods have suffered over the years, since Krogh's pioneering work [4,5], from the lack of experimental data of sufficient detail and precision to critically test the proposed mathematical models. Thus, for about a half-century the Krogh approach to mathematical simulation was used almost exclusively. Krogh and his colleagues treated the diffusion problem in the tissue surrounding a "typical" capillary under the tacit assumption that the blood in the capillary was well-mixed radially. This assumption is equivalent to assuming that the resistance to oxygen transport in the tissue is of dominant importance over that within the lumen of the microvessel. In the last two decades it has come to be understood that the intraluminal resistance to oxygen transport is of at least equal importance as that in the surrounding tissue, and thus cannot be neglected in an accurate simulation [6–11]. The focus of this paper is on these important intraluminal transport processes — including the oxygen transport characteristics of suspensions of erythrocytes in

solutions of hemoglobin or hemoglobin polymer used in hemoglobin-based oxygen carriers.

A brief outline of both experimental and mathematical methodologies will be given. We then give a discussion of "flow regimes". Significant oxygen transport occurs in microvessels of a very large range of diameters in the microvasculature. Very different flow regimes are found in vessels of different diameters, and very different approaches are required for mathematical simulation of the oxygen transport in the different regimes. Finally, we present results of experimental measurements in two categories: (1) results are given from oxygen release experiments on suspensions of erythrocytes in buffer containing various amounts of extracellular hemoglobin flowing in a 25 μm diameter microvessel; and (2) we present results of oxygen uptake and release experiments in a newly-developed 10 μm diameter capillary system — for hemoglobin solutions and red cell suspensions.

Methods

The experimental system is illustrated schematically in Figure 1. The apparatus has been described in detail recently [12].

The system includes a "capillary" (either 25 μm or 10 μm diameter) embedded in a thin silicone rubber film. Silicone rubber was selected because of its high permeability to oxygen combined with sufficient optical clarity to permit spectroscopic determination of hemoglobin oxygen saturation. A silicone rubber film containing a single capillary is mounted on the stage of a microscope. The capillary is cannulated at each end and perfused with red blood cell suspensions, hemoglobin solutions, polyhemoglobin solutions or mixtures thereof. A dual wavelength microspectrophotometric method is used to determine oxygen saturation of the sample at various axial positions along the capillary. The sample in the feed reservoir is equilibrated with a humidified gas of the desired oxygen tension and suffused with the same gas (often either air or nitrogen) while in the feed reservoir. The capillary film is suffused with a humidified gas of an independently-selected oxygen tension. In a typical oxygen uptake experiment the feed reservoir contains a deoxygenated sample, and the gas suffusing the capillary film is air. In a typical oxygen release experiment the feed reservoir contains an oxygen-saturated sample and the gas suffusing the capillary film is nitrogen.

The specimens used in the experimental work discussed here all contained a total of 10 g/dl of hemoglobin — whether in the form of erythrocytes, hemoglobin in solution, or mixtures. The erythrocytes were drawn from human subjects, washed and resuspended as described previously [12]. The suspended cells had a p_{50} of 27 ± 1 mmHg and a Hill coefficient of approximately 2.65. The hemoglobin solution was a purified bovine material prepared by Biopure Corporation with a p_{50} of 25 ± 1 mmHg and a Hill coefficient of approximately 2.65.

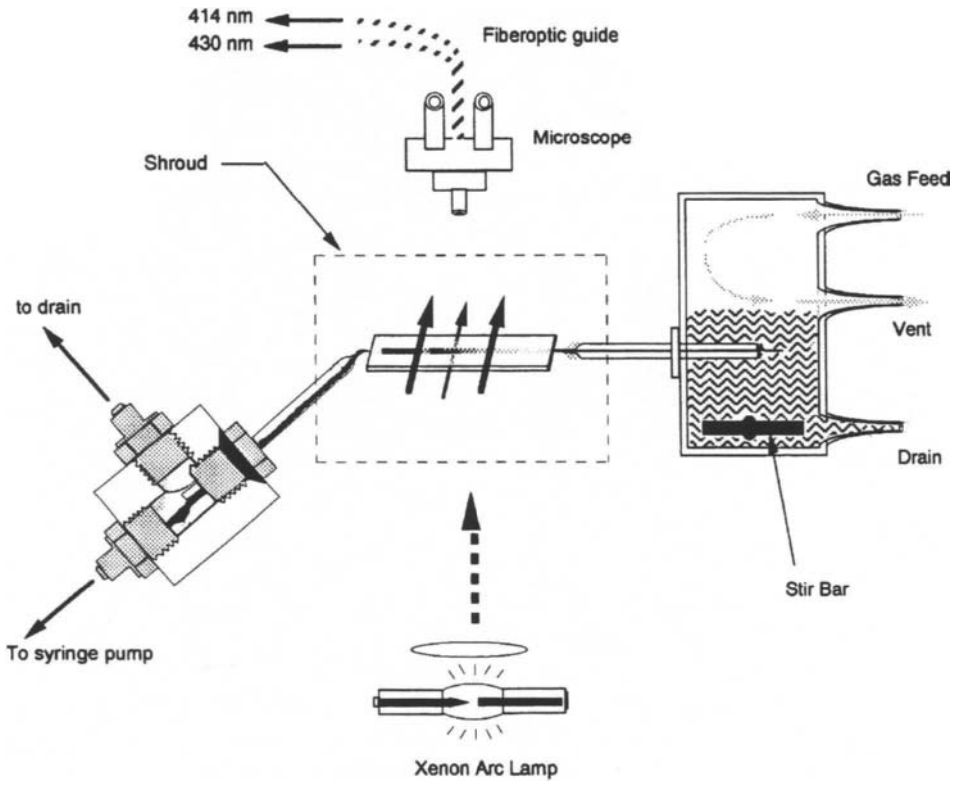

Fig. 1. Schematic illustration of the experimental system. Hemoglobin containing samples (erythrocyte suspensions, solutions of hemoglobin or hemoglobin polymer, and mixtures) are passed through a single capillary lumen (either 10 or 25 μm diameter) formed in a thin film of silicone rubber. Experiments simulating oxygen uptake or release are carried out by independently controlling the oxygen tension of the feed sample and that of the gas suffusing the silicone rubber film. Oxygen saturations of the hemoglobin were measured spectrophotometrically at several axial positions along the capillary. (From Page et al., [12]).

In our prior mathematical work we treated two cases:

1. The partial differential equations governing oxygen transport in hemoglobin solutions were solved for conditions used in experiments in the artificial capillary system, and the solutions were shown to agree well with the corresponding experimental measurements [8,13]. This good agreement validated the experimental system, since the theory of oxygen transport in homogeneous hemoglobin solutions has been well developed for many years.
2. The partial differential equations needed to treat oxygen transport in red cell suspensions were developed and solved for conditions used in experiments in the artificial capillary system, and the solutions were shown to agree well with the corresponding experimental measurements [8,14]. The complicated processes

governing red cell motion and transport defy exact simulation, and some simplifying assumptions are required to obtain a system of equations that are mathematically tractable. This requirement of simplifying assumptions necessitates the experimental validation of the theory. Such validation has been missing in much of the prior work.

The third stage in the model is in progress. It involves adapting the two prior cases described above to treat the case of interest in blood substitutes: red cells suspended in a hemoglobin solution. The adaptation has been found to be more challenging than simply applying the equations of the prior work. One of the complications is that transport in the suspending medium is much more important when the suspending medium contains dissolved oxyhemoglobin. Convective currents in the suspending medium associated with cell–cell interaction must be taken into account in some cases where extracellular hemoglobin (or polyhemoglobin) is present.

Flow regimes

The character of the flow and, hence, the transport processes in the microcirculation are highly dependent on the diameter of the microvessel. We recognize three distinctly different flow regimes as outlined below and illustrated in Figs. 2–4. Figure 2 is a micrograph of erythrocytes flowing in a 25 μm diameter vessel in the artificial capillary system. The figure gives a visual detail of the relationship between the erythrocyte dimensions and the conduit diameter. In vessels in this (arteriolar) size

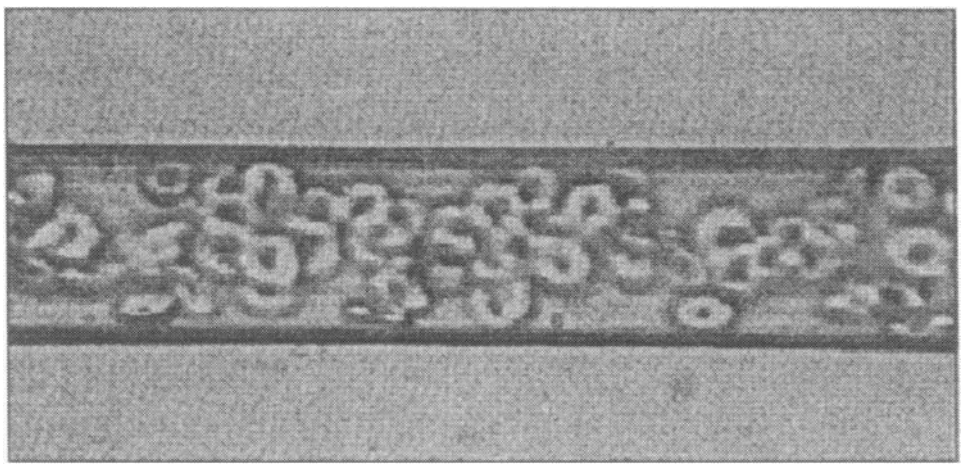

Fig. 2. Erythrocytes flowing in a 25 μm diameter artificial capillary. This image is of an erythrocyte suspension in buffer flowing through the capillary slowly at a reduced hematocrit to permit visualization of the individual cells.

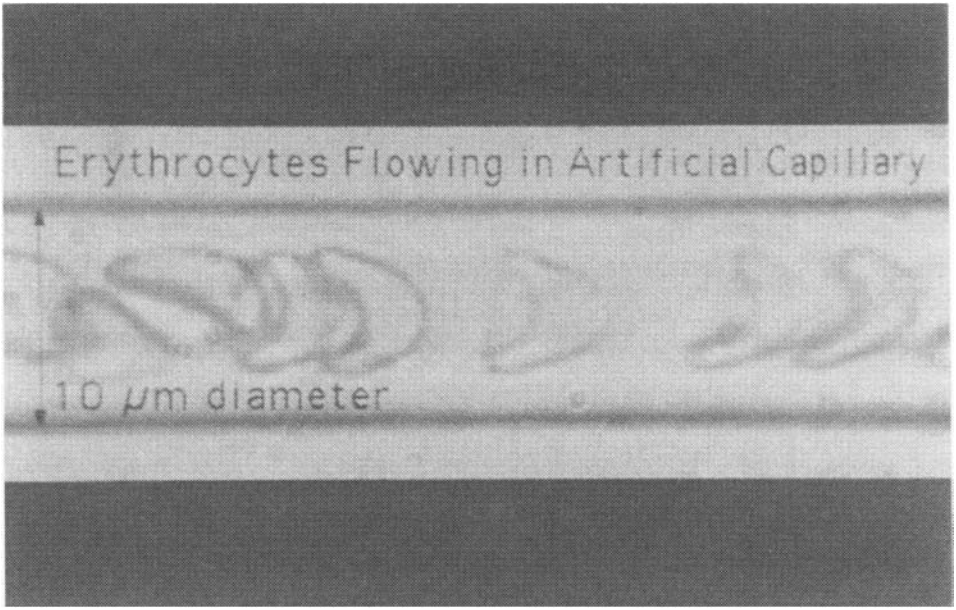

Fig. 3. Erythrocytes flowing in a 10 μm diameter artificial capillary. This image is of erythrocytes suspended in buffer flowing through the capillary at a rate of 5.5 μl/h.

range (approximately 20–100 μm diameter) we have shown that the idealization of a local hematocrit that varies continuously with radial position is useful. The variation in the hematocrit across the vessel is taken into account. This approach has been successful in mathematical simulation of the results of oxygen transport experiments in the vessel diameter range of 20–100 μm [8,13].

Figure 3 is a photograph of erythrocytes flowing in a 10 μm diameter vessel in the artificial capillary system. Here we see that the flow is of an entirely different character: the cells consistently pass through the vessel in single file. Thus, the concept of a local hematocrit that varies continuously with radial position is not applicable. Very little work has been done on vessels in this particular size range. One study in this regime was performed by Wang and Popel [15] based on the cell shapes calculated for an 8.24 μm diameter vessel by Zarda et al. [16]. Later in this paper we will present the first in vitro experimental measurements of oxygen transport in this flow regime.

Figure 4 is a schematic representation of the two regimes discussed above plus the important third regime: that of the true capillaries with diameters in the 4–6 μm range. In the 25 μm diameter capillary the idealization of a local hematocrit that varies with radial position has been found to be useful. This local hematocrit is represented in the Figure by the curve h(r). Note that h(r) is shown to be zero in a thin layer of thickness near the capillary wall, in accordance with experimental observations. In the smaller vessels the erythrocytes tend to move in single file as suggested in

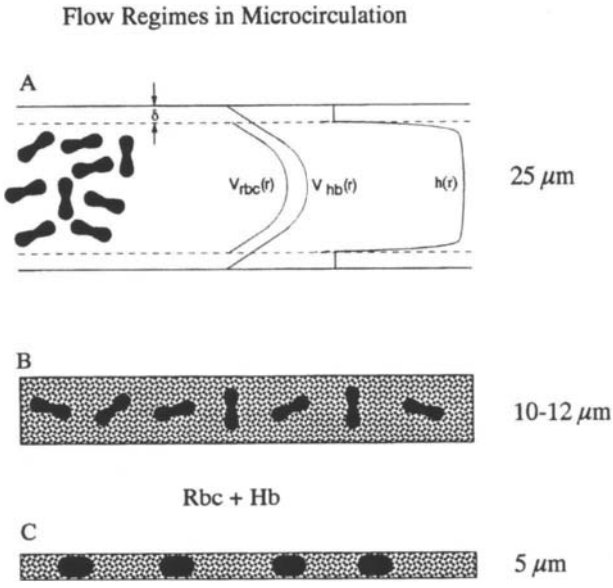

Fig. 4. Schematic illustration of three distinct flow regimes in the microcirculation. The upper sketch, "A", is a schematic representation of the flow illustrated in Fig. 2. The middle sketch, "B" is a schematic representation of the flow illustrated in Fig. 3. The lower sketch, "C", is a schematic representation of the flow in true capillaries (of diameter 4–6 μm). The dark dots in the background of sketches "B" and "C" are intended to suggest the presence of a soluble hemoglobin-based oxygen carrier in the suspending medium.

the sketches "B" and "C". In the smallest vessels, "C", the erythrocytes are strongly deformed to approximate a cylindrical shape, and the layer of suspending medium between the erythrocytes and the capillary wall is comparatively small [17,18]. Additional discussion of these flow regimes and the prior work on oxygen transport by both experimental and theoretical means has been reviewed by Hellums et al. [1].

Oxygen release results in arteriolar-sized vessels

Figure 5 shows oxygen release results from a 25 μm diameter artificial capillary in a simulation of the performance of a blood substitute. In this case the specimens enter the capillary fully oxygen saturated. The capillary film is suffused with nitrogen. Thus, each specimen becomes deoxygenated as it flows through the capillary. All specimens have the same total hemoglobin content. The upper curve gives results for an erythrocyte suspension in buffer. The bottom curve gives results for a solution of bovine hemoglobin. The intermediate curves are intended to simulate the clinical use of a blood substitute — representing the replacement of either 10% percent (labeled Rbc/Bhb 9–1) or 50% (labeled Rbc/Bhb 1–1) of the erythrocytes with extracellular hemoglobin.

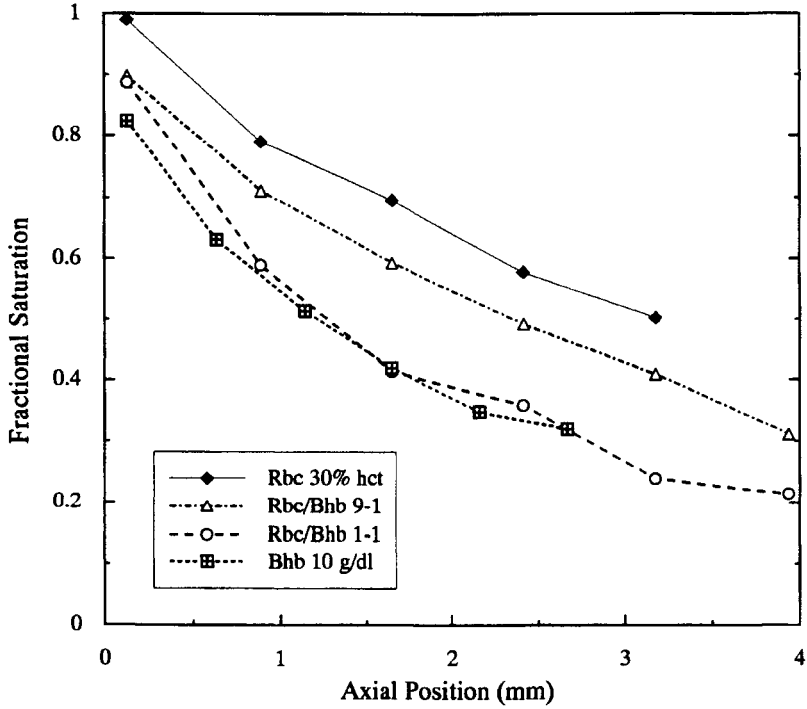

Fig. 5. Oxygen release experiments with and without use of extracellular hemoglobin. Results are presented in terms of hemoglobin oxygen saturation at various axial positions along the 25 μm diameter microvessel. All samples were fully oxygen saturated in the feed reservoir and released oxygen while flowing through the microvessel. The top curve is for an erythrocyte suspension. The bottom curve is for a bovine hemoglobin solution. The two intermediate curves are for suspensions of erythrocytes in hemoglobin solutions. All of the samples were of the same hemoglobin content. The points denoted 9–1 have 90% of the hemoglobin within the erythrocytes. The points denoted as 1–1 have equal amounts of hemoglobin within and without the erythrocytes.

All specimens started with a fractional oxygen saturation of 1.0. Thus, it can be seen that the hemoglobin solution delivers oxygen much more effectively than the erythrocyte suspension: the change in oxygen saturation is strikingly higher for the hemoglobin solution. Replacement of half of the erythrocytes with hemoglobin solution yields oxygen delivery that is virtually as efficient as the pure hemoglobin solution. Furthermore, replacement of only 10% of the erythrocyte hemoglobin gives a substantial increase in oxygen delivery over the case of erythrocytes alone.

Results for the 10 μm diameter artificial capillary

We have recently extended our experimental investigations with 10 μm diameter silicone rubber capillaries. The results for a series of oxygen uptake and release

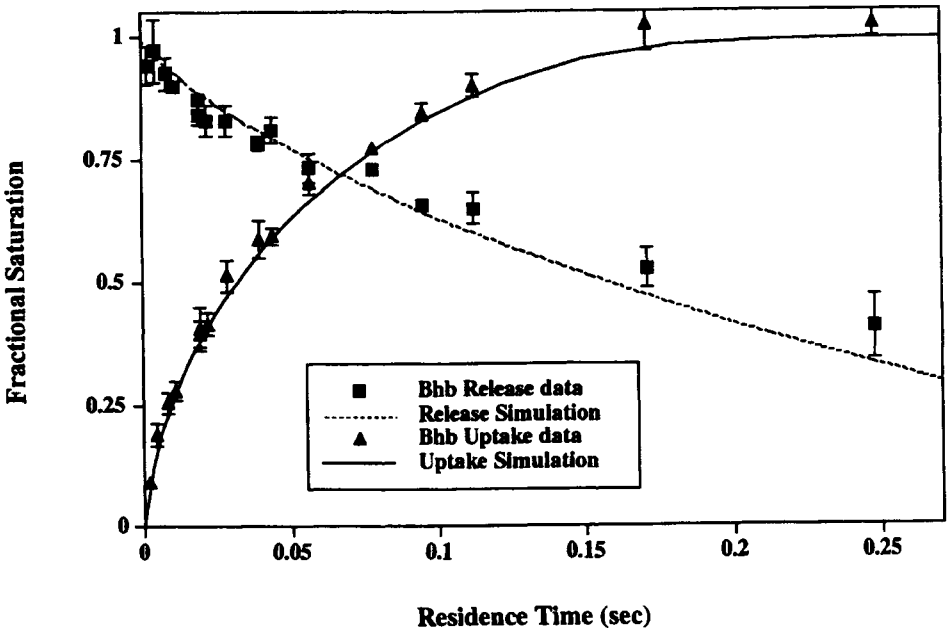

Fig. 6. Hemoglobin solution oxygen uptake and release experiments for a 10 μm diameter capillary. Experimental determinations of hemoglobin oxygen saturation are presented as a function of residence time in the capillary. The data points represent measurements at four axial positions and at four different flow rates. The error bars denote the standard deviation for three replicate experiments. The curves are from theoretical calculations.

experiments are given in Fig. 6 for flow of a hemoglobin solution. The curves on the figure are from our theoretical calculations, and the points are from the experimental measurements. There is excellent agreement between the theory and experiments. The abscissa in the figure is apparent residence time in the capillary rather than axial position. The data represent results of measurements at four axial positions at four different flow rates. The results for the different flow rates are integrated to a single smooth curve when presented as a function of residence time. Notice that in the uptake experiments the specimens became fully saturated in approximately 0.2 s. The corresponding time for full saturation in the 25 μm diameter capillary was of the order of 1.0 s. The more rapid oxygenation in the smaller vessels is to be expected for several reasons: one of which is the fact that the ratio of the vessel surface area for transport to the vessel volume is inversely proportional to vessel diameter.

Figure 7 gives our first, preliminary results on erythrocyte suspensions in the 10 μm diameter capillary. In this case the oxygen saturation is given versus axial position. The apparent residence time is easily calculated from the formula $t = (LA)/Q$ where "t" denotes the apparent residence time, "L" denotes the axial position, "A" denotes the vessel cross sectional area, and "Q" denotes the volumetric flow rate. The

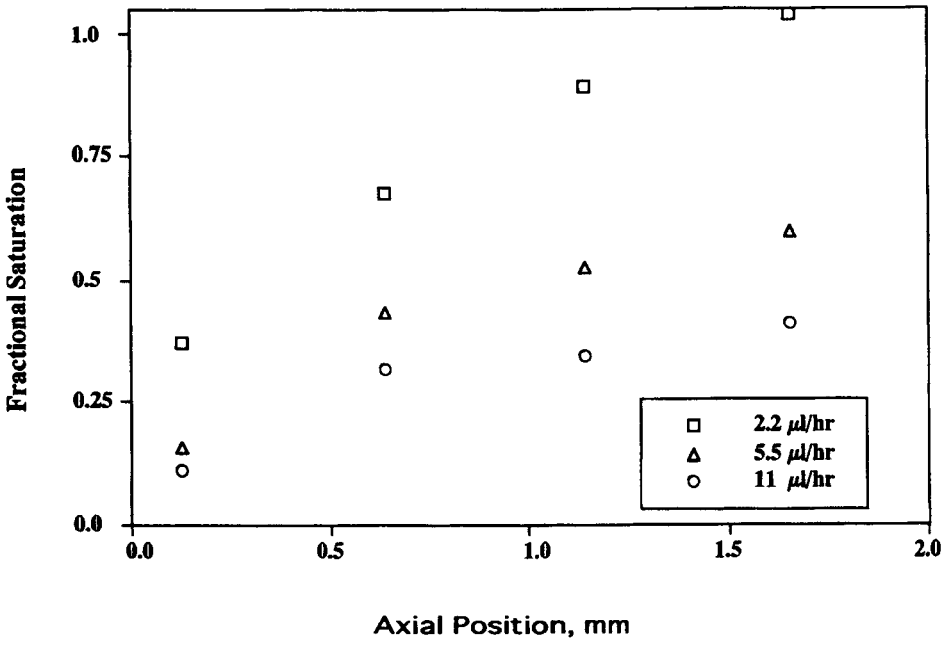

Fig. 7. Oxygen uptake experiments for erythrocyte suspensions flowing in a 10 μm diameter capillary. Experimental determinations of hemoglobin oxygen saturation are presented as a function of axial position in the capillary for three different suspension flow rates as indicated.

residence times for the erythrocytes are of the same order of magnitude as for the hemoglobin solutions (Fig. 6). However, as was the case for the arteriolar-sized artificial capillary, the oxygen transport is significantly more rapid in the hemoglobin solution.

These results for the 10 μm diameter capillary represent substantial progress. However, there is need for much more research of both the experimental and theoretical nature on oxygen transport in vessels of this and smaller diameter.

Concluding remarks

Oxygen transport occurs in microvessels of a wide range of diameters in the human circulation. As a result, there are three distinct flow regimes with different mechanisms of transport. The arteriolar regime (represented schematically as the top sketch of Fig. 4) has been studied in our prior work [8,12,13,19]. This paper presents the first in vitro results in the intermediate regime (middle sketch in Fig. 4). Substantially more research is needed on both theoretical and experimental simulation of oxygen transport in both this intermediate regime, and in the true capillary regime (bottom sketch of Fig. 4). The summary given below is based on our research in the arteriolar size regime, and on our preliminary results in the intermediate regime.

The artificial capillary system gives us the unique capability to simulate oxygen uptake and release in vitro in experiments in which all flow, geometric, and transport parameters are controlled and are known accurately.

The good agreement between the experimental and mathematical results tend to validate both the experimental methodology and the mathematical model.

The results confirm that hemoglobin solutions are much more efficient than red cell suspensions of the same hemoglobin content in oxygen transport over a wide range of vessel diameters. Replacement of even a small fraction of the erythrocytes with extracellular hemoglobin-based oxygen carrier yields substantial enhancement in oxygen transport.

Acknowledgments

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 12

Tissue Oxygen Delivery and Tissue Distribution of Liposome Encapsulated Hemoglobin

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Introduction

Research efforts to develop an adequate red cell substitute have focused primarily on oxygen carrying formulations based on either hemoglobin or perfluorochemicals [1–3]. Although this chapter will emphasize methods to assess hemoglobin-based red cell substitutes, many of the approaches described could also be applied to perfluorocarbon-based red cell substitutes.

An important concern related to the use of these various red cell substitutes is the duration of their effectiveness for transporting oxygen after administration. For obvious reasons, the development of red cell substitutes with increased physical persistence in the circulation has been a long-standing objective of red cell substitute development. Researchers have implemented various strategies in order to meet this objective. For example, intra- and intermolecular cross-linking of hemoglobin, increased the circulation half-life of hemoglobin to 6–12 h in various animal models as compared to 0.5–1.5 h for unmodified hemoglobin [4]. Another technique which has been used to increase the circulation persistence of hemoglobin is conjugation with polyethylene glycol (PEG) [5,6]. PEG is a biologically inert compound regularly used in both cosmetic and pharmaceutical preparations and is considered safe for use by the FDA [7]. The encapsulation of hemoglobin in liposomes is yet another approach that results in prolonged circulation persistence of the encapsulated hemoglobin and, at the same time, shifts its clearance route from the kidneys to the reticuloendothelial system (RES) [8,9].

A second important factor in prolonging the functionality of a red cell substitute is the state of the hemoglobin molecule. The hemoglobin molecule has to remain in a functional state after administration, otherwise it will not be able to deliver oxygen. To evaluate the ability of a red cell substitute to carry and transport oxygen to the tissues after in vivo infusion, several factors must be considered. To state it simply, the

hemoglobin molecule must be able to on-load oxygen when passing through the capillaries of the lungs and off-load oxygen when passing through the capillaries of the tissues.

The ability of a red cell substitute to on-load oxygen in the lungs is generally known as its oxygen carrying capacity. Oxygen carrying capacity is determined not only by the total amount of hemoglobin present in circulation, but also the state of the hemoglobin molecule, which must remain in reduced form since the oxidized form of hemoglobin (met-hemoglobin) does not bind oxygen. All hemoglobin-based red cell substitutes are susceptible to met-hemoglobin conversion during storage and after infusion into the body [10]. Oxygen carrying capacity is also effected by affinity of the hemoglobin molecule which must be high enough that oxygen will be on-loaded in the lungs.

The other very important requirement of a red cell substitute is that it must be able to off-load oxygen at the tissues. The conformation of the hemoglobin molecule determines the affinity that hemoglobin has for the oxygen molecule. If the oxygen affinity of the hemoglobin molecule is too high, the amount of oxygen released by the hemoglobin molecule at the tissue level will be reduced so that tissue oxygen delivery will be impaired. Although it has generally been assumed that the oxygen affinity of hemoglobin in a red cell substitute should be similar to that of the host red cells for adequate oxygen delivery, there has been little scientific evidence to support this assumption. Recently, much debate has been initiated concerning what is the ideal affinity for the hemoglobin in a red cell substitute [11]. The ideal affinity for a red cell substitute may be different from the ideal affinity of hemoglobin contained in a red cell, because oxygen leaving a red blood cell has to pass through the diffusive barrier of the low oxygen soluble plasma. This diffusive plasma barrier does not exist for red cell substitutes which are homogeneously dispersed in the plasma, and may only partially exist for hemoglobin encapsulated in liposomes. A red cell substitute with a low affinity could potentially release oxygen excessively rapidly in the precapillary arterioles which could result in autoregulatory vasoconstriction [12]. Also complicating this picture is that low affinity hemoglobin molecules tend to be less stable in plasma, and are more rapidly converted to met-hemoglobin which cannot carry oxygen [13].

All of these three previously mentioned factors, (1) circulatory persistence, (2) oxygen carrying capacity and (3) oxygen release at the tissue level, are important aspects of an effective red cell substitute, but assessing and quantitating each of these individual characteristics in vivo has been difficult. Until recently, most studies performed to demonstrate efficacy of a red cell substitute have relied on monitoring animal survival after 95% or complete exchange transfusions with a red cell substitute. This approach is limited, however, because it does not allow for the accurate comparison between various red cell substitute formulations and is not applicable to studies in primates and humans.

Our laboratory has developed several techniques, based on radioisotopic tracers for evaluating the in vivo circulatory persistence and functional capacity of red cell substitutes. These include the use of (1) the imaging radionuclide, technetium-99m

(^{99m}Tc), for the non-invasive determination of liposome encapsulated hemoglobin (LEH) biodistribution and circulatory persistence and (2) oxygen-15 (^{15}O) labeled molecular oxygen for in vivo determination of oxygen carrying capacity and tissue oxygen delivery. We have used these techniques to evaluate various red cell substitutes and as tools to assist in the development of new red cell substitutes. These tools, particularly ^{15}O, also offer a promising means to increase our basic understanding of oxygen transport in a variety of pathophysiologic conditions.

Our research, using these analytic techniques, has principally focused on the development of second and third generation liposome encapsulated hemoglobin (LEH) formulations. The initial area of our emphasis was on determination of the biodistribution of LEH and assessment of its circulatory persistence using the ^{99m}Tc-liposome label. Encapsulation of hemoglobin in liposomes significantly increased the circulation half-life of hemoglobin from less than 2 h to approximately 20 h [8]. The clearance was biphasic with a rapid phase of uptake by the macrophages in the liver followed by a slower phase uptake by the splenic macrophages. This line of research has lead us to further attempt to increase hemoglobin circulatory persistence by modifying the surface characteristics of the liposome and, therefore, create a third generation red cell substitute (second generation LEH) with a circulation half-life significantly greater than 20 h.

Modification of the surface of LEH with polyethylene glycol (PEG) appeared to be the most promising strategy to further increase the circulation persistence of LEH. PEG-surface modification of standard liposome formulations increases their persistence in the circulation by decreasing their rate of removal by the RES [14–16]. However, a previous attempt to prolong the circulation of LEH by surface modification with 5 mol% distearoyl phosphoethanolamine polyethylene glycol (PEG-PE) did not increase the circulation persistence of the LEH over LEH without PEG surface modification [17]. It now appears that the precise mol% of PEG-PE added to the LEH formulation is critical in order to successfully increase the circulation persistence of LEH. Studies in our laboratory were performed in an attempt to determine if the addition of a higher 10 mol% PEG-PE with a PEG-headgroup of greater molecular weight (5000 vs 1900) would increase the circulation persistence and change the tissue biodistribution of LEH. This LEH formulation containing 10% PEG-PE was chosen because it had been quite successful in prolonging the circulation persistence of empty liposomes studied as a diagnostic imaging agent for cardiac imaging [18].

In this study, PEG-LEH was produced by microfluidization following rehydration of a dried lipid film consisting of 50 mol% distearoyl phosphatidylcholine (DSPC), 39 mol% cholesterol, 10 mol% distearoyl phosphoethanolamine-*N*-[polyethylene glycol-5000] (PEG-PE) and 2 mol% α-tocopherol (mole ratio 50 : 38 : 10:2) with α,α-crosslinked human hemoglobin (Bionetics, Rockville, MD) containing 30 mM reduced glutathione (Sigma, St. Louis, MO), 10 mg/ml human serum albumin and 9% sucrose [19]. The final PEG-LEH preparation was tested as previously described [19,20] and displayed the following characteristics: unimodal size distribution,

193 nm; phospholipid content, 110.5 mM; hemoglobin content, 1.2 g/dl; endotoxin level, 18 EU/ml; and sterility, no growth for 14 d in thioglycolate media at 37°C.

The circulation persistence and biodistribution of PEG-LEH was monitored using the ^{99m}Tc-liposome label [8,18,21,22]. ^{99m}Tc is the most common isotope used for human clinical imaging studies in nuclear medicine because of its ideal energy characteristics and low dosimetry. Using ^{99m}Tc for labeling liposomes allows for not only tissue sample counting after the animals are sacrificed, but also allows for a dynamic tracking of the LEH biodistribution over time in the same animal using non-invasive scintigraphic imaging. This labeling technique was originally developed for labeling LEH because previous methods of labeling liposomes with ^{99m}Tc only labeled the surface of the liposome and the ^{99m}Tc became dissociated from the liposomes after injection [22,23]. This resulted in large amounts of ^{99m}Tc appearing in the kidneys and the urine located in the bladder which are not representative of the distribution of the LEH. We were able to develop a completely new method of labeling liposomes using the lipophilic ^{99m}Tc chelator, hexamethylpropyleneamine oxime (HMPAO) (Ceretec, Amersham, Arlington Hgts, IL). HMPAO is a brain imaging agent in nuclear medicine and is commercially available. This labeling technique was found to depend on glutathione which was contained in the LEH as an anti-oxidant. This labeling technique has been shown to be widely applicable for tracking of all types of liposome preparations and liposomes labeled with this technique are currently being developed as diagnostic imaging agents in nuclear medicine by several groups [18,22,24,25].

In our study, we labeled PEG-LEH (4 ml) using ^{99m}Tc-HMPAO by this new method. Male New Zealand White rabbits (2.5–3.0 kg) were anesthetized and infused with a total dose of PEG-LEH equivalent to 25% of their circulating blood volume. Images and blood samples were acquired during the first 2 h and at 24 and 48 h after which the animals were sacrificed and tissues taken for counting in a scintillation well counter for determination of the biodistribution.

The images acquired at 2, 24 and 48 h can be seen in Fig. 1 and are compared to an image of ^{99m}Tc-labeled red blood cells (^{99m}Tc-labeled RBCs). It can be seen that the PEG-LEH persists in the blood pool by noticing the similarity of the PEG-LEH images to those of ^{99m}Tc-labeled RBCs. The activity in the region of the heart represents blood pool activity since there is very minimal uptake of LEH in the heart muscle. As can be seen, significant activity remains in the region of the heart even at 48 h which provides a visual demonstration of the long circulatory persistence of the PEG-LEH formulation. Minimal activity can be seen in the spleen over the entire 48 h time period. Figure 2 illustrates the organ distribution of PEG-LEH at 48 h as a percentage of injected dose (ID) determined from the tissue counts taken at the time of sacrifice. At 48 h, greater than 50% of the activity remains in circulation and the extrapolated circulation half-life was 65 h. This contrasts remarkably with several previous non-PEG surface modified LEH preparations that had circulation half-lives of less than 20 h [8,26]. The majority of the PEG-LEH is cleared by the liver. At 48 h, 13% of the activity is in the liver while another 10% has already been cleared from the

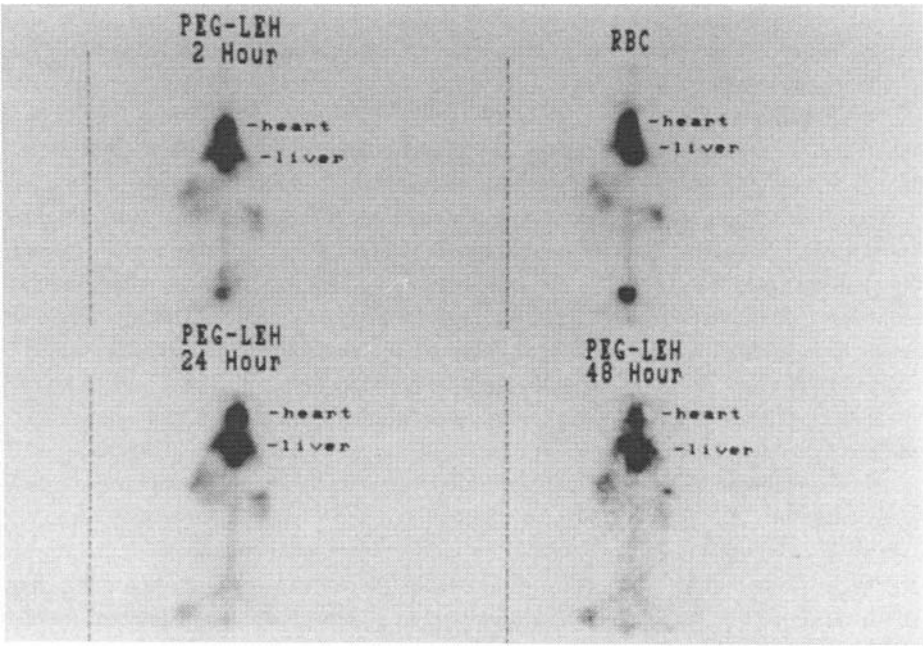

Fig. 1. Gamma camera images of rabbits acquired after a 25% topload infusion of ^{99m}Tc-labeled PEG-LEH. The images were taken at 2 h (upper left-hand corner), 24 h (lower left-hand corner) and 48 h (lower right-hand corner) after infusion of ^{99m}Tc-PEG-LEH. For comparative purposes an image of ^{99m}Tc-labeled red blood cells (RBC) acquired after equilibration at 20 min is shown (upper right-hand corner). The 2 h image ^{99m}Tc-PEG-LEH is very similar to the ^{99m}Tc-RBC images.

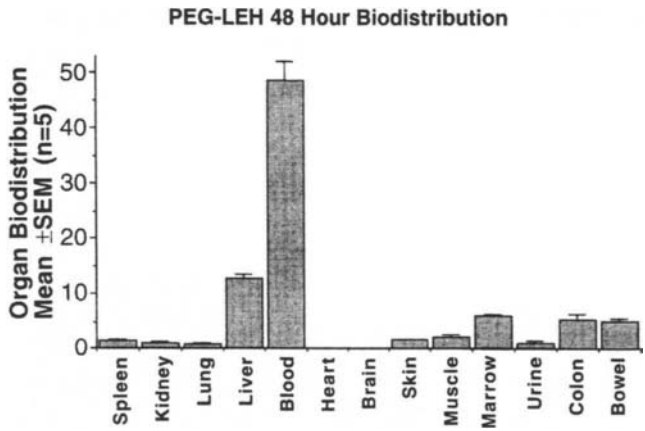

Fig. 2. The 48 h organ biodistribution for ^{99m}Tc-PEG-LEH on a % ID/organ basis obtained by tissue sampling at necropsy. The blood has 4 times more ^{99m}Tc-PEG-LEH (51.27 ± 3.44% ID) than the liver (12.69 ± 0.70% ID). 5% ID is found in the bone marrow, the small bowel and the colon. Only minimal amounts are noted in the other organs.

liver and is in the colon and other bowel. Only 2% of the activity is located in the spleen. In a previous study with non-PEG surface modified LEH, 18% of the LEH was taken up by the spleen [8].

From this study, it can be seen that PEG-modification of LEH has much potential to prolong the circulatory properties of LEH. The hemoglobin concentration of this current PEG-LEH formulation is low and methods to increase its hemoglobin concentration need to be developed. One possible method to increase the hemoglobin content of LEH is by the use of other LEH manufacturing techniques. The dehydration–rehydration technique has recently been described as a method that results in LEH with a hemoglobin content of over 10 g/dl [27]. Tsuchida et al. has also described a LEH formulation with a fairly high hemoglobin concentration in this range [9].

Further studies will also need to address whether the addition of PEG to LEH increases the toxicity of the PEG-LEH formulation at sites of clearance such as the liver and the spleen. The oxygen carrying and oxygen delivery characteristics of this PEG-LEH will also need to be determined. Methods to assess these characteristics will be discussed in the following paragraphs.

In addition to methods for studying the circulation persistence of LEH, we have developed several approaches for determination of the oxygen carrying and delivery efficacy of a variety of hemoglobin-based red cell substitutes using the radioisotope ^{15}O. ^{15}O-molecular oxygen is used for monitoring oxygen transport (1) from the lungs to blood for determination of oxygen carrying capacity or (2) to the tissues for measurement of the oxygen extraction fraction. These techniques can determine the oxygen carrying capacity and tissue delivery specifically by the red cell substitute in the presence of other oxygen carriers, namely RBCs by measuring annihilation photons emitted from ^{15}O either up-loaded from the lungs or off-loaded to the tissues. This specific ability to separately measure the function of the red cell substitute in the presence of RBCs contrasts with many other techniques that measure tissue oxygenation without regard to the specific agent that transported the oxygen. These ^{15}O techniques include frozen myocardial spectroscopy, microelectrodes, phosphorescence quenching, magnetic resonance spectroscopy, near-infrared spectroscopy, NADH fluorescence and electron spin resonance which have been summarized for their usefulness in assessing oxygenation either directly or indirectly by Wagner and Schied [28].

Oxygen-15 is a positron-emitting radioisotope with a short half-life of 2 min, and is routinely produced in a cyclotron in the form of molecular oxygen (^{15}O–O_2) by bombarding 8 MeV deuterons to a gas target containing 99% nitrogen and 1% carrier oxygen [29]. This ^{15}O–O_2 can then be converted to ^{15}O-labeled water ($H_2{}^{15}O$) by mixing the ^{15}O–O_2 with hydrogen gas and passing the gas mixture over a palladium catalyst heated to 150°C or ^{15}O-labeled carbon monoxide ($C^{15}O$) by passage through a 1000°C furnace with activated carbon [29].

Like all positron emitters, ^{15}O undergoes a decay scheme in which a positron or positively charged electron is produced by transformation of a proton in the nucleus to a neutron and a positron [30]. This positron is ejected from the nucleus and travels

only a short distance in the tissue before it combines with an ordinary electron, leading to an annihilation event. The products of this annihilation are two 511 KeV photons which leave the site in opposite directions 180° apart. It is these photons that we are measuring in our studies either with a scintillation well counter or scintillation detector probe. Oxygen-15-radiopharmaceuticals are used frequently in positron emission tomography (PET), especially in studies to map a sensory or motor event to a precise location in the brain [31,32].

Oxygen-15-labeled molecular oxygen can be bound to hemoglobin either as part of the blood substitute or native RBC, and monitored directly to determine oxygen transport properties. The short half-life of ^{15}O allows studies to be repeated in the same animal at 15 min intervals so that the release kinetics of oxygen from hemoglobin in red cell substitutes can be compared to that of RBCs. The ^{15}O–O_2 released from the hemoglobin enters the tissues from the blood and participates in oxidative phosphorylation to form ^{15}O-labeled water ($H_2{}^{15}O$). The $H_2{}^{15}O$ then mixes with the intracellular water pool in the tissues and also gradually returns to circulation at a rate based on the organ blood flow rate [33].

An outline of the method we have developed to measure oxygen uptake from the lungs by the red cell substitute or its carrying capacity with ^{15}O–O_2 is shown in Fig. 3.

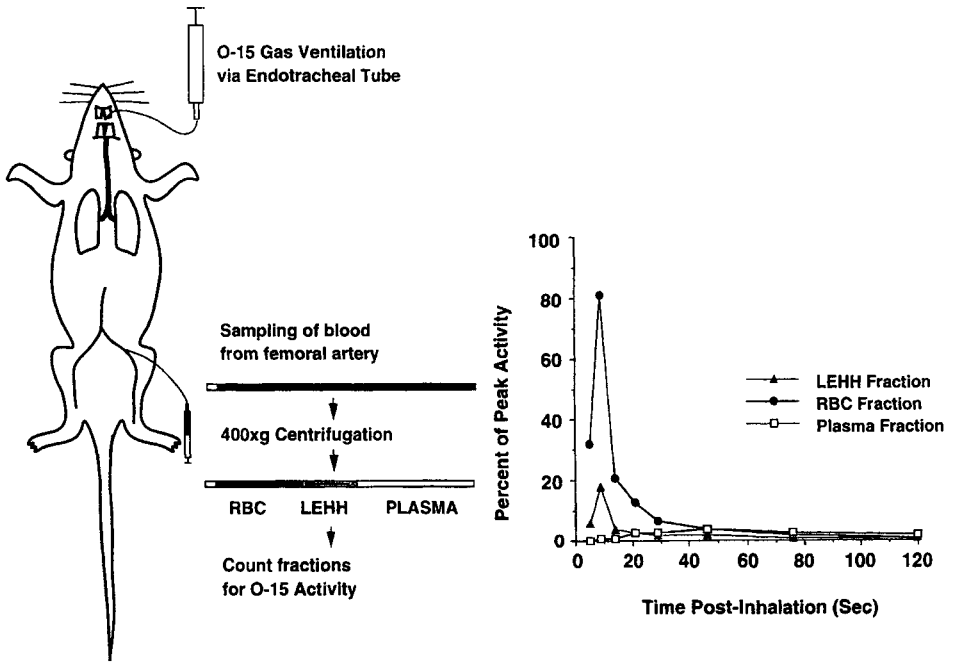

Fig. 3. Schematic representation of the experimental protocol for determining the oxygen carrying capacity of a red cell substitute using ^{15}O–O_2.

Male Sprague Dawley rats (250 g) with an indwelling femoral artery catheter were anesthetized with sodium pentobarbital (30 mg/kg, intraperitoneally) and intubated. Once anesthetized, the rats underwent a 40% exchange transfusion. After a 15 min stabilization period, a 4 mCi dose of ^{15}O–O_2 gas in 8 ml was drawn into a syringe from a Scanditronix MC-17 cyclotron and was insufflated into the lungs of a rat. Serial blood samples were immediately collected from a femoral artery catheter into capillary tubes. These tubes were then spun in a microhematocrit centrifuge. The plasma, red cell substitute and RBC fractions were rapidly separated and counted in a Canberra scintillation well counter. The radioactivity data was decay-corrected and then plotted. A typical data set of decay-corrected ^{15}O-activity obtained from serial arterial samples of a rat receiving LEH is shown in Fig. 3. The ^{15}O-activity peaks in the LEH and RBC fractions within 10 s and then is rapidly removed during the next 30 s. During this same period, the low initial ^{15}O-activity in the plasma increases, which represents conversion of the ^{15}O–O_2 to metabolic water ($H_2{}^{15}O$). Peak activity of each fraction was used to determine the oxygen carrying capacity of the hemoglobin in the red cell substitute in relationship to the hemoglobin in the remaining RBCs using algebraic expressions as previously detailed in our published work [34].

These insufflation experiments with ^{15}O–O_2 can be repeated at various times as often as every 15 min until the red cell substitute is totally removed from the circulation. Figure 4 shows an example of this type of study in which three different red cell substitutes, (1) free unencapsulated bovine hemoglobin (SFHS); (2) liposome encapsulated bovine hemoglobin (LEBH); and (3) liposome encapsulated human hemolysate (LEHH), were studied. The human hemolysate contained naturally occurring anti-oxidants and met-hemoglobin reductase that appeared to protect the hemoglobin and greatly reduced its conversion to met-hemoglobin. The oxygen

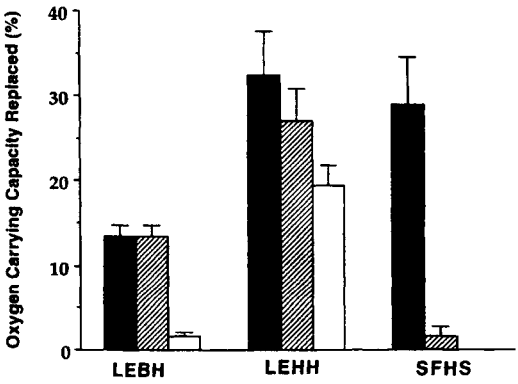

Fig. 4. Oxygen carrying capacity replaced by the red cell substitute as a percentage of the oxygen carrying capacity of the red cells withdrawn from circulation for LEH containing bovine hemoglobin (LEBH), LEH containing human hemolysate (LEHH) or free bovine hemoglobin (SFHS) over a 24 h period after exchange transfusion in rats.

carrying capacity of LEHH was greatly retained at 24 h and was lost only due to the removal of the LEHH from the circulation by the RES. The LEBH totally lost its ability to carry oxygen by 24 h even though one half of the infused hemoglobin was still in circulation. This loss in oxygen carrying capacity was due to the conversion of its remaining hemoglobin to met-hemoglobin. The SFHS had lost most of its oxygen carrying capacity at 3 h due to rapid removal and met-hemoglobin conversion of hemoglobin in circulation. This study emphasizes the importance of developing methods to retain the hemoglobin of red cell substitutes in a functional reduced state. Since human hemolysate is not a defined media, approaches are currently underway investigating the coencapsulation of enzymes and anti-oxidants with hemoglobin in a liposome in order to create an even better artificial red cell in which the hemoglobin is protected in vivo [35,36].

The method we have developed to determine the extraction fraction of oxygen from a red cell substitute by the brain is outlined in Fig. 5. ^{15}O–O_2 bubbled red cell substitute is injected into the carotid artery of rabbits and recording made from detectors position over the brain to determine the extraction fraction of oxygen from the red cell substitute. Male New Zealand White rabbits (3 kg) were initially anesthetized with ketamine/xylazine (40 mg/kg: 5 mg/kg) intramuscularly and heparinized with 1000 U/kg. Rabbits were maintained in a stable state of anesthesia for the duration of the study by continuous intravenous infusion of ketamine/xylazine (0.24 mg/kg: 0.03 mg/kg) in Lactated Ringer's. The left carotid artery was catheterized using a T-connection to ensure an easy access with no restriction of blood flow to the brain. The head of the rabbit was then placed between two bismuth germanium oxide (BGO) scintillation detector probes with the probes collimated inside a lead

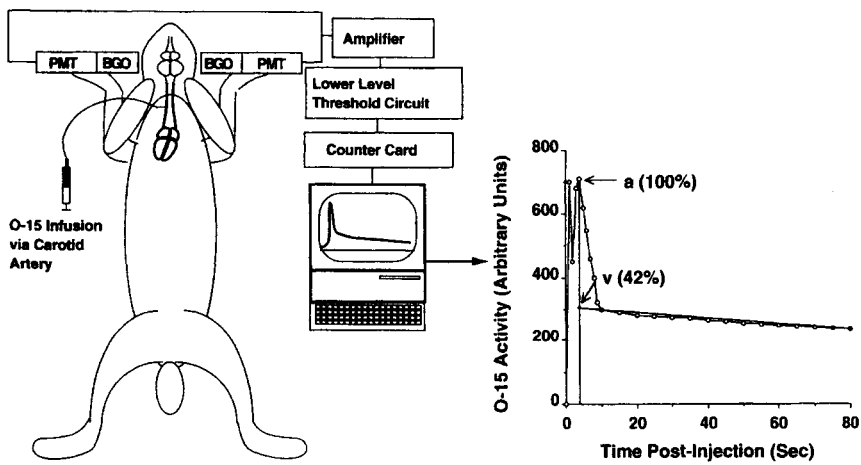

Fig. 5. Diagram of the experimental protocol for measuring the oxygen extraction fraction of a red cell substitute labeled with ^{15}O–O_2.

cylinder with 1.5 inch thick walls and aligned with the brain for maximum sensitivity. Once stabilized, the rabbit was given a bolus injection (0.3 ml) of either $^{15}O_2$-labeled-autologous RBC (150 mCi; 39 mg hemoglobin), ^{15}O-labeled-LEH containing α,α-crosslinked hemoglobin (30 mCi; 3.8 mg hemoglobin) or ^{15}O-labeled α,α-crosslinked hemoglobin (30 mCi; 3.8 mg hemoglobin). RBC and red cell substitute samples were labeled with ^{15}O by bubbling each sample with ^{15}O–O_2 in vitro. The ^{15}O-activity detected from the brain by the probes was processed and stored on a computer. Following decay correction, each curve was plotted as a function of the fraction of counts of the peak activity. A typical curve showing ^{15}O-activity collected by the probe plotted versus time of injection is shown in Fig. 5. The initial spike represents the ^{15}O-activity in the major vessels of the brain that pass by the probes prior to reaching the capillaries of the brain. The second peak represents the total ^{15}O–O_2 activity reaching the capillaries of the brain. A fraction of this peak activity is extracted by the brain and converted to $H_2{}^{15}O$, which then gradually exits the brain over a 10 min period. This extracted fraction is known as the oxygen extraction fraction and is calculated as the ratio of the back extrapolation of the water washout curve (v) over the maximum value of the second peak (a) [37] (See Fig. 5).

Figure 6 shows curves obtained using the scintillation detector probes to determine the behavior of the ^{15}O-radioisotope as a part of $H_2{}^{15}O$ or when in the form of ^{15}O–O_2 or $C^{15}O$ bound to autologous RBC. These curves are compared to ^{15}O–O_2 bound to PEG-LEH. These data clearly show the distinction between the behavior of ^{15}O when associated with these different molecules. As a part of $H_2{}^{15}O$, the ^{15}O freely flows into the brain tissue and mixes with the intracellular water pool before gradually exiting the brain tissue so that $> 90\%$ is extracted by the brain in the first pass. This high first pass extraction allows $H_2{}^{15}O$ to serve as a marker of tissue perfusion while the rate of removal of the $H_2{}^{15}O$ from the brain is dependent on the rate of blood flow to the brain. In contrast to $H_2{}^{15}O$, the ^{15}O associated with $C^{15}O$ remains tightly bound to the RBC hemoglobin and does not extract into the brain tissue, but remains associated with the blood and is rapidly carried into other parts of the body until it equilibrates with the blood pool (approximately 10% of peak counts). The $C^{15}O$ therefore serves as a marker of the transit time of red blood cells through the brain. Both of these situations are very different compared with what happens with the ^{15}O–O_2 associated with RBC hemoglobin. In the case of ^{15}O–O_2-labeled RBC, approximately 40–50% of ^{15}O extracts into the brain tissue before being metabolized to $H_2{}^{15}O$, which is slowly removed from the brain at the same rate as if it had been perfused with $H_2{}^{15}O$. This slow removal can be observed in Fig. 6. These results confirm that this detector system developed for small animals behaves as expected for each agent and in the same manner as was originally described in humans by Ter-Pogossian. These previous studies by Ter-Pogossian were the first to use positron emittors in medical studies and from which eventually developed positron emission tomography (PET) imaging [32,37].

Further studies using this same detector system have shown that the hemoglobin labeled with ^{15}O–O_2 as a component of either autologous RBC, LEH containing α,α-

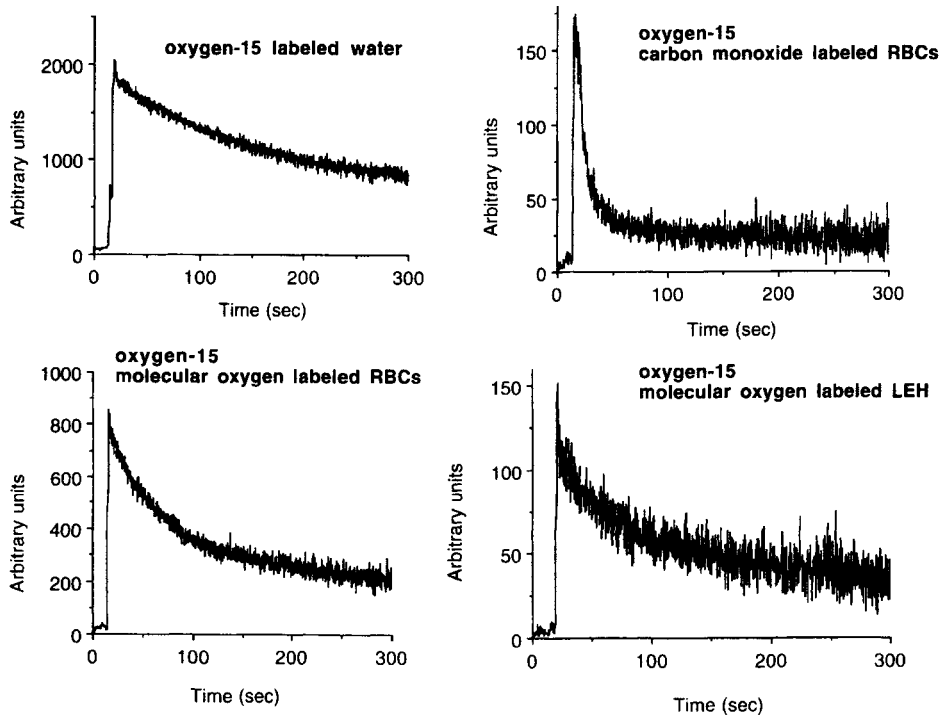

Fig. 6. ^{15}O–O_2 activity curves recorded over the head of a rabbit in which the carotid artery was injected with $H_2{}^{15}O$ (upper left hand corner), $C^{15}O$-labeled RBC (upper right hand corner), ^{15}O–O_2-labeled RBCs (lower left hand corner) or ^{15}O–O_2 labeled PEG-LEH (lower right hand corner).

crosslinked human hemoglobin or free α,α-crosslinked human hemoglobin and which has similar affinities behave in a similar fashion and have similar extraction fractions. This technique will be useful for determining the effect of hemoglobin oxygen affinity on tissue oxygen delivery [11]. ^{15}O–O_2 also offers much promise as a tool to investigate the effects of various pathophysiologic conditions on oxygen delivery, oxygen utilization, mean transit time through the brain, and cerebral blood flow and may provide new understandings of these conditions.

The studies presented in this chapter outline a variety of novel radioisotopic techniques applied to the study of red cell substitutes. The described techniques that have been adapted for use in small animals have been very beneficial in surveying different hemoglobin-based red cell substitutes for effective oxygen carrying and delivery properties. These methods have many advantages including the use of physiologic and non-toxic radiotracers to monitor circulatory persistence and oxygen delivery of red cell substitutes as well as the ease with which these methods can be applied to humans receiving red cell substitutes in a clinical setting.

Acknowledgments

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 13

Polymeric Biodegradable Hemoglobin Nanocapsule as a New Red Blood Cell Substitute

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Introduction

Early artificial red blood cells

In the first study on artificial red blood cells, polymeric membranes are used to replace red blood cell membranes [1]. The first artificial red cell membranes are coated with an ultrathin layer of organic liquid. This allows the retention of 2,3-DPG while at the same time allows the rapid equilibration of oxygen and carbon dioxide across the membranes. The oxygen dissociation curve is therefore similar to that of red blood cells [1]. Red blood cell enzymes from hemolysate like carbonic anhydrase [2] and catalase [3] are retained in the artificial cells with enzyme activities. Thus the encapsulated catalase acts as an antioxidant against the toxic effects of hydrogen peroxide in acatalesemic mice that has an inborn error of metabolism in catalase [3]. The artificial red blood cells do not have blood group antigens on the membrane, as a result, they do not form aggregates in the presence of blood group antibodies [5].

The single major remaining problem of these early encapsulated hemoglobin is their rapid removal after intravenous infusion. We found that they have to be less than 1 μm to avoid being trapped in the lung capillaries [4,5]. Even then, they are removed by the reticuloendothelial systems. Our studies of why red blood cells can circulate for so long show that removal of sialic acid from the red blood cell membranes results in their rapid removal from the circulation [4–6]. We therefore prepared artificial red blood cells with surface modifications. This included the use of other synthetic polymers [2–9] including membranes with different surface charge and polysahccharide surfaces as sialic acid analogs. We also studied the use of crosslinked hemoglobin membrane [2,5,10]. Some of these improved the circulation time. However, the circulation time was still not enough for practical applications. The smallest microcapsules used at this time have a mean diameter of about 1 μm.

Cross-linked hemoglobin

In an attempt to decrease the diameters and prepare more flexible artificial red blood cell membranes we started to cross-link the surface layers of hemoglobin of hemoglobin emulsions [2,5]. The first cross-linker used was sebacyl chloride [2,5,7]. Decreasing the emulsion diameter resulted in the preparation of smaller artificial red blood cells. With further decrease in diameter, all the hemoglobin in the emulsion is cross-linked into polyhemoglobin. We have also studied the combination of different ratios of hemoglobin and polymer in the crosslinking process with the formation of conjugated hemoglobin. We also looked at the use of glutaraldehyde to cross-linked hemoglobin and enzymes inside artificial red blood cells to form stable soluble preparations [10]. Continuing decrease in the number of hemoglobin crosslinked have now resulted in soluble polyhemoglobin that are being used in clinical trials.

Artificial red blood cells with lipid membranes

Mueller et al. reported that by modifying our method [12] of preparing microencapsulated hemoglobin, they can prepare lipid membrane artificial red blood cells [11]. However, the resulting membranes are not sufficiently stable. We therefore prepared artificial red blood cells with lipid-protein and lipid-polymer membranes [5,12]. Djordjevich and Miller reported in 1980 that they can prepare smaller 0.2 μm diameters lipid membrane vesicles encapsulating hemoglobin [13]. This substantially increased the circulation time, although the circulation time was still rather short. Many groups have since then carried out research to successfully improve the preparation and the circulation time [14-18]. Modifications of surface properties including surface charge and the use of sialic acid analogues have further improved the circulation time. The average half-time in the circulation is now more than 30 h. It is possible to replace most of the red blood cells in rats with these artificial red blood cells.

The two groups of Rudolph in the USA and Tsuchida in Japan have made extensive progress. They are collaborating with many groups using their preparations. Studies by several groups show that there are no adverse changes in the histology of brain, heart, kidneys and lungs of experimental animals. Preclinical studies are being finalized towards clinical trials.

Biodegradable polymeric hemoglobin nanocapsules

More recently, we have been investigating the use of biodegradable polymeric hemoglobin nanocapsules. This is to prepare microencapsulated hemoglobin with the following properties:

1. Increase stability in storage and after infusion.
2. Decrease the potential effects on the reticuloendothelial systems.
3. Avoid lipid peroxidation.

4. Solve the problem of methemoglobin formation.
5. Increase the concentration of hemoglobin encapsulated.

Choice of biodegradable polymer

We have been using biodegradable polymer, e.g. polylactic acid, for the encapsulation of hemoglobin, enzymes and other biologically active material since 1976 [19–21]. With the availability of methods to prepare microcapsules of nanometer diameters, we started to prepare hemoglobin nanocapsules of less than 0.2 μm mean diameter using polylactic acid membrane and other biodegradable polymers (Fig. 1) [22–26].

Biodegradable polymers for forming nanocapsule membranes have to be non-toxic and degradable in the body into nontoxic degradation products. Polyesters, like polylactic acid, polyglycolic acid and their copolymers polylactoglycolides degrade finally into lactic acid and glycolic acid that are normal human metabolites. The rate of degradation can be adjusted by variations in molecular weight, particle size, ratio of the copolymer. Our rationale for the selection of polyesters like polylactic acid, polyglycolic acid and polylactoglycolides is because of the reasons given above and also because of extensive clinical experience in their uses in other clinical applications. Since 1982, these polyesters have been used extensively as Dexon-S suture in human with no adverse or toxic response. There have also been other uses and surgical implantations with no reported toxicity.

Properties of hemoglobin nanocapsules

Electron microscopic examinations show that they are spherical and homogeneous. Their mean diameter can be as small as 80 nm and as large as 180 nm or more. The membrane thickness is about 5–15 nm. The diameter and size distribution of the

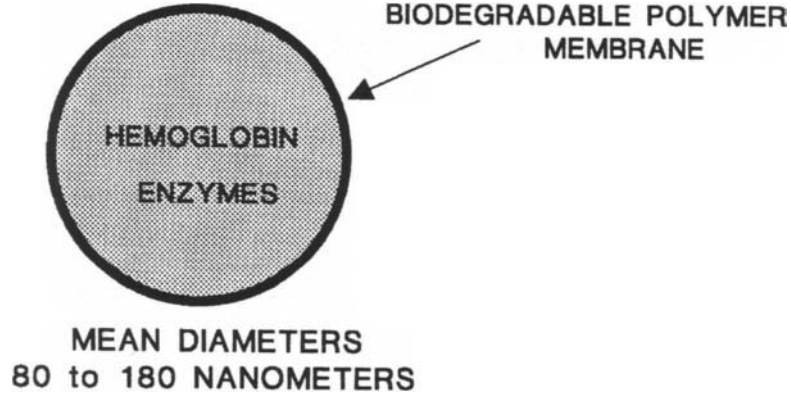

Fig. 1. Schematic representation of a polylactic membrane nanocapsule containing hemoglobin and enzymes.

biodegradable hemoglobin nanocapsules are determined by using the Nicomp Size Analyzer (Model 370). The average particle sizes of biodegradable nanocapsules containing hemoglobin is dependent on the formula used for preparation. An unimodal distribution is obtained for all samples (Fig. 2). With different preparation process and different polymers, the mean diameters of biodegradable hemoglobin nanocapsules can be as low as 80 nm. Nanocapsule hemoglobin content can be as high as 15 g/dl suspension comparable to 100 ml in blood (Fig. 3). The characteristics are: hemoglobin concentration of 10.97 g/dl to 15 g/dl; polylactide concentration of 1.2 g/dl; phospholipid concentration of 0.6 g/dl; specific gravity (22°C) of 1.0047; viscosity (37°C) of 3.7–3.8 cP. Steady shear viscosity of the suspension of the nanocapsules containing hemoglobin was measured with a Wells-Brookfield Syncro-Lectric Microviscometer (Model LVT) equipped with a 0.80° cone (Model CP-40). Shear rates were from 45 to 450 s^{-1} at 22°C. The steady shear viscosities of the biodegradable polymer hemoglobin nanocapsules at 22°C are 6.5 cP at shear rates of 45 s^{-1} and 90 s^{-1} and 5 cP at shear rate of 225 s^{-1}. At present, the hemoglobin encapsulation efficiencies before optimization range from 13% to 29% of the starting quantities of hemoglobin, depending on the polymer used. Higher amount of hemoglobin is encapsulated with poly(D.L)lactic acid. The ratio of hemoglobin/phospholipid/polymer for the preparation with hemoglobin of 10.97 g/dl suspension is 15/1/2. Higher concentrations of up to 15 g/dl suspension has also been prepared more recently (Fig. 3). This approaches the hemoglobin concentration of whole blood.

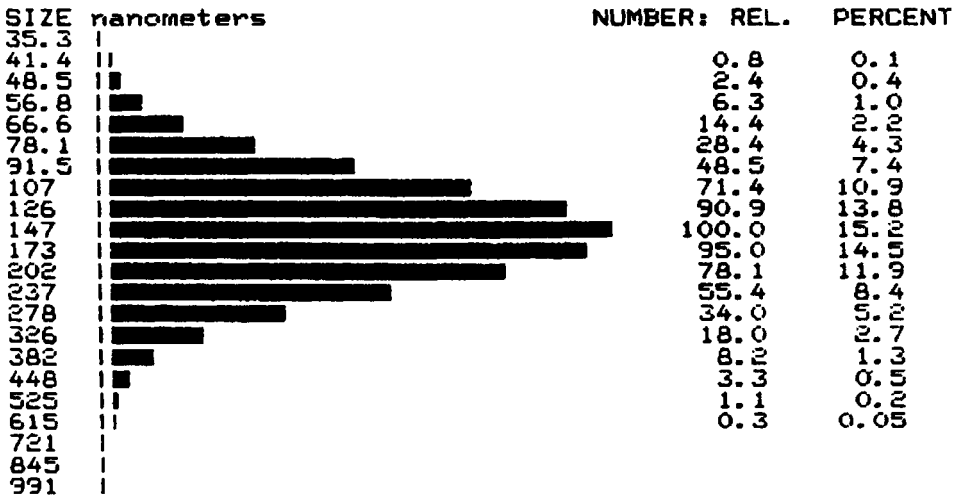

Fig. 2. Typical size distribution of biodegradable polymer hemoglobin nanocapsules. Nicomp Size Analyzer (Model 370). With permission from: Yu, WP, and TMS Chang, Artificial Cells, Blood Substitutes and Immobilization Biotechnology, an International Journal, 24:169–184, 1996. Courtesy of Marcel Dekker Publisher.

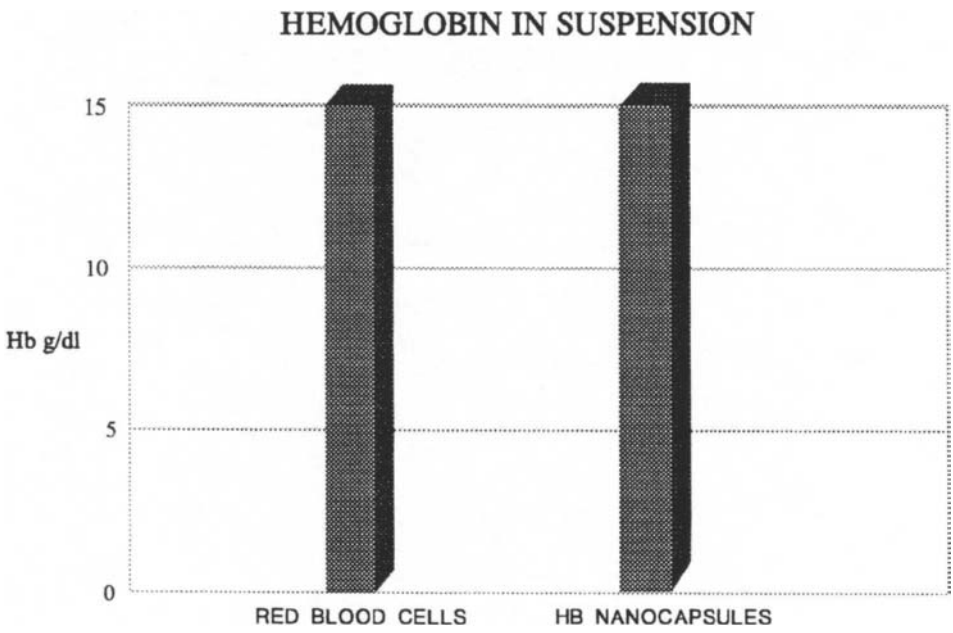

Fig. 3. Hemoglobin concentration in blood and hemoglobin nanocapule suspension. For hemoglobin nanocapsules, hemoglobin concentration of up to 15 gm/dl can be prepared. Reprinted with permission from: Chang, TMS. Artificial Cells, Blood Substitutes and Immobilization Biotechnology, an International Journal, 25:1–24, 1997. Courtesy of Marcel Dekker Inc.

Oxygen affinity, Hill coefficient and Bohr effect

The oxygen dissociation curve is determined using the TCS Hemox analyser (TCS Medical Products Co., USA). There is no significant difference between hemoglobin nanocapsules and free bovine hemoglobin. Hill coefficient is 2.4–2.9. These results show that the procedure of preparation does not have adverse effects on the hemoglobin molecules. In the physiological pH range, oxygen affinity of polymer membrane containing hemoglobin changed with pH. The Bohr effect is about −0.22 to −0.24.

Enzymes and multienzymes

Artificial cells have been prepared to include multienzyme systems with cofactor recycling [27]. A number of enzymes normally present in red blood cells can be encapsulated within these biodegradable polymer hemoglobin nanocapsules [22,23,26]. This may be important for example in stabilising the hemoglobin and also in preventing adverse effects of oxygen radicals. As will be discussed later, we have also encapsulated the methemoglobin reductase system and showed that this can convert methemoglobin to hemoglobin [26] (Fig. 4). Nanocapsules may improve on

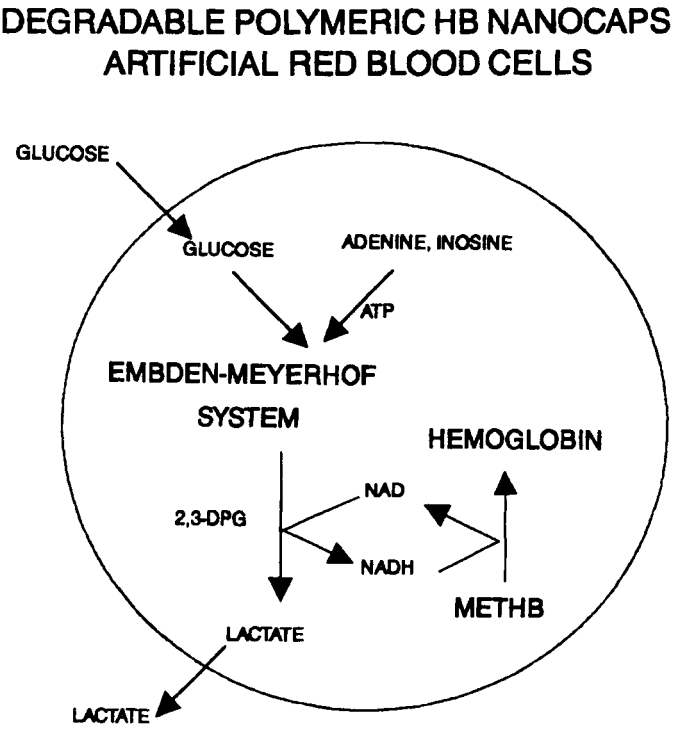

Fig. 4. Hemoglobin nanocapsules containing multi-enzyme system to reduce methemoglobin. Reprinted with permission from: Chang, TMS. Artificial Cells, Blood Substitutes and Immobilization Biotechnology, an International Journal, 25:1–24, 1997. Courtesy of Marcel Dekker Inc.

the problem related to methemoglobin reductase system in standard encapsulated hemoglobin systems. Since lipid vesicles are not permeable to glucose, the required glucose has to be added in high concentrations into the lipid vesicles. In the case of nanocapsules, the biodegradable polymeric membranes can be made permeable to glucose and other molecules. This allows us to prepare hemoglobin nanocapsules containing the methemoglobin reductase system to function as shown in Fig. 4. External glucose can diffuse into the nanocapsules. Products of the reaction can diffuse out and therefore do not accumulation in the nanocapsules to inhibit the reaction. In vitro study shows promising results in the conversion of methemoglobin to hemoglobin.

Circulation half-life

Circulation half-life of hemoglobin nanocapsules is evaluated on male rats. Cannulation of the femoral artery and vein are carried out. Each rat is injected with 1/3 of its

blood volume by top loading. The survival time of the nanocapsules in circulation is followed. Preliminary studies show that incorporation of PEG markedly increased the circulation time of these hemoglobin nanocapsules to a clinically useful duration. We are continuing with more detailed studies on this.

Feasibility analysis based on results obtained

By assuming that all the polymer added is incorporated into the nanocapsule membrane, the maximal amount of polymer per 500 ml of hemoglobin nanocapsule is shown in Fig. 5. The total amount of membrane material is much less than the total membrane material in hemoglobin lipid vesicles. The membrane material of hemoglobin nanocapsules as shown in Fig. 4, is made up mostly of biodegradable polymer. Since polymer is stronger than lipid and is also porous, much less membrane material is required. For a 500 ml suspension, the total lactic acid produced is 83 mEq. This is far less than the normal resting body lactic acid production (1000–1400 mEq/d). The maximal body capacity to breakdown lactic acid is 7080 mEq/d. Thus, 83 mEq is equal to 1% of this. Furthermore, the polylactic acid in the hemoglobin nanocapsules is biodegraded over a number of days and therefore, there is an even smaller amount released per day for each unit of hemoglobin nanocapsules. This with the other results obtained so far encourages us to develop this approach for potential clinical applications. Further details are available elsewhere [28,29].

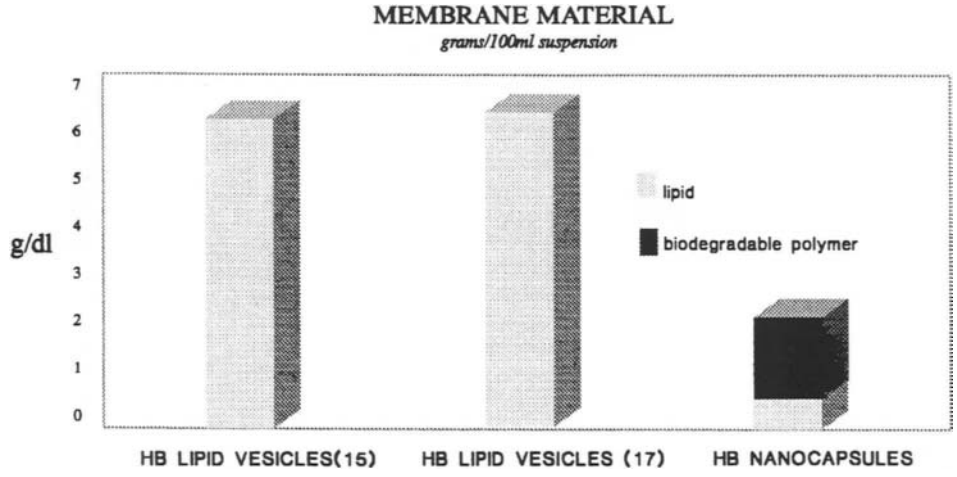

Fig. 5. Membrane materials of hemoglobin lipid vesicles and hemoglobin nanocapsules. Reprinted with permission from: Chang, TMS. Artificial Cells, Blood Substitutes and Immobilization Biotechnology, an International Journal, 25:1–24, 1997. Courtesy of Marcel Dekker Inc.

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 14

Evaluation of the Oxygen Transporting Capability of Hemoglobin Vesicles

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Introduction

Enormous efforts have been made to develop red cell substitutes, especially those utilizing Hb to overcome problems associated with blood transfusions such as the necessity for blood typing, fear of infectious disease, alloimmunization, and graft-versus-host diseases, difficulties in storage, etc. [1–3]. Hemoglobin vesicles (HbV) or liposome-encapsulated hemoglobin (LEH) are red cell substitutes which have a cellular structure of phospholipid vesicles [4–7]. On the other hand, in acellular Hb solutions such as chemically modified Hb and recombinant Hb, clinical trials are underway at present. Though various modification has been made to overcome the issues of acellular Hb, inevitable difference between cellular red blood cell and acellular Hb has been discussed relating to irregular physiological responses such as vasoconstriction and autoregulation, Hb toxicity, and so on [8–11]. HbV with a cellular structure is expected to make solutions of those issues. In order to increase the oxygen carrying capability of HbV as an oxygen carrier, we have to consider the followings. (1) The regulation of oxygen affinity of HbV, (2) increase in the concentration ratio of Hb to total lipid components of the vesicles ([Hb]/[Lipid]), (3) chemical stabilization of an encapsulated Hb solution, namely the suppression of metHb formation (metHb has no ability to bind oxygen), and (4) high dispersibility in blood circulation. Those are summarized schematically in Fig. 1. In this chapter, the new preparation method which relates to those points, especially the construction of artificial metHb reduction system, and effect of polyethyleneglycol-modified HbV in blood circulation are described as well as in vivo evaluation of the efficacy of HbV.

New preparation method of HbV

We have been studying about how to encapsulate the concentrated Hb with thin lipid layers from the control of molecular assembling, because phospholipid vesicles with diameter larger than 0.1 μm are multilamellar vesicles and encapsulation of with

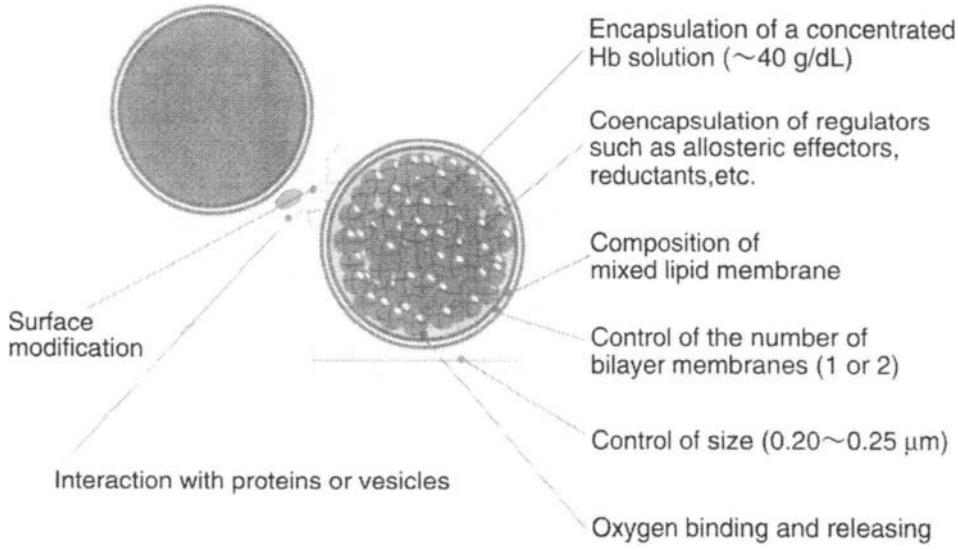

Fig. 1. Illustration of characteristics of Hb-vesicles.

concentration higher than 35 g/dl was difficult [12]. It was clarified that the pH value of Hb solutions influenced the properties of resulting HbV such as [Hb]/[Lipid] ratio, p_{50} value, and the rate of metHb formation as shown in Fig. 2 [13]. The [Hb]/[Lipid] of HbV showed the maximum value of 1.8 at pH 7.0, relating to the isoelectric point of Hb ($pI = 7.02$) at the preparation of HbV. Because the negative ζ-potential of Hb at the pH values higher than the pI causes the electrostatic repulsion between Hb molecules and the negative surface of the vesicle and results in the decrease of the [Hb]/[Lipid]. On the other hand, the lower pH reduces the negative surface potential of the vesicle and increases the number of bilayer membranes of HbV [12]. It is generally known that the metHb formation is facilitated at lower pH because of proton oxidation, however, higher pH increases the oxygen affinity to a higher value than that of RBC ($p_{50} = 27$ mmHg). Therefore, the pH value of the Hb solution should be adjusted to 7.0 in the preparation of HbV, and it is more desirable for the pH value to be adjusted to 7.4 where HbV functions as an oxygen carrier [13].

Generally, the pH control of the inner aqueous phase of HbV is difficult by simply changing the pH in the outer aqueous phase because the permeability of counter ions of H^+ and OH^- through the bilayer membrane is very low. Carbon dioxide decreases the pH of an aqueous solution in proportion to the partial pressure. From the pH measurement of the inner aqueous phase of vesicle, it was clarified that the pH change of the inner aqueous phase could be controlled by changing the partial pressure of carbon dioxide. Because a hemoglobin solution shows a buffer effect against the pH change, the relationship between pH and pCO_2 was obtained with various concentrations of Hb and various temperatures [13].

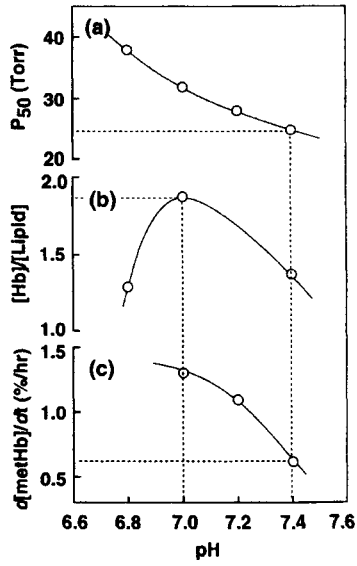

Fig. 2. Influence of the pH of Hb solutions at the preparation of HbV on (a) the oxygen affinity (p_{50}), (b) encapsulation efficiency ([Hb]/[Lipid]), and (c) the rate of metHb formation (d[metHb]/dt) of the resulting HbV.

The pH of the Hb solution used for the preparation of HbV was adjusted to 7.0 from 7.4 using N_2/CO_2 mixed gases, and HbV with a high [Hb]/[Lipid] was prepared. The pH of HbV was adjusted to 7.4 by removing the dissolved CO_2 under the reduced pressure. The resulting pH-controlled HbV with p_{50} of 27 mmHg showed a low rate of metHb formation.

Construction of artificial metHb reduction system

The common issue of Hb-based red cell substitutes is the relatively rapid metHb formation during storage or blood circulation [14,15] due to the absence of metHb reduction systems originally existing in a red blood cell. The systems include enzymatic reduction such as NADH-cytochrome b_5 and NADPH-flavin, direct reduction by glutathione (GSH) and ascorbic acid, and scavengers for active oxygen species such as superoxide disumutase (SOD) for $O_2^{\cdot -}$ and catalase for H_2O_2. HbO_2 dissociates into not only Hb and O_2, but also metHb and $O_2^{\cdot -}$. by one electron transfer [16–18]. When Hb is autoxidized to metHb, it loses its oxygen binding ability. However, the percentage of metHb in RBC is maintained at less than 0.5% of the total Hb by the systematic reduction.

Utilization of the activities of metHb reduction systems remaining in the RBC is one method to lower the metHb formation rate of encapsulated Hb [19]. However, their activity would change with the conditions of outdated red blood cell from which

hemolysate is prepared, the amounts of remaining substances, and encapsulation procedure using high shear stress. Virus inactivation using heat treatment is impossible in this case. Moreover, the mechanism of metHb reduction systems is complicated and influenced by many unknown factors. In our purification method of Hb with heat treatment, these enzymes and chemicals are denatured and then removed [20]. High purity of Hb is very important to prepare HbV containing highly concentrated Hb with high reproducibility and high efficiency. Therefore, the construction of a metHb reduction system by coencapsulation of an appropriate amount of reductants is required.

There are many reductants which can reduce the metHb to deoxyHb under anaerobic condition. However, a few of them can suppress the metHb formation of HbV under aerobic conditions; some enhance the metHb formation. This occurs because, at the beginning, there is a small percentage of metHb to be reduced in the HbV and encapsulated reductants are generally autoxidized faster than the rate of metHb formation under aerobic conditions. Furthermore, active oxygens generated from such autoxidation oxidize the Hb to metHb [21,22]. The coencapsulation effect of a series of thiols (cysteine, Cys; glutathione, GSH; homocysteine, Hcy; and acetylcysteine, Acy) was studied as reductants for HbV. Hcy and GSH showed a good suppressive effect on metHb formation, while Cys adversely accelerates the metHb formation at a rate twice that of the Hb solution without any reductants, and Acy showed no change [23,24]. At least two contributions should be considered to explain this result; one is a positive contribution of thiols to reduce the metHb; the other is a negative contribution by autoxidation of the thiols, which destroys their ability, and generated active oxygens enhance the metHb formation. The significant suppression of metHb formation of HbV by the coaddition of SOD and catalase with Cys indicates that Cys is easily oxidized by oxygen and simultaneously generates a large amount of active oxygens. On the other hand, Hcy and GSH showed the effective suppression of the metHb formation because their low rates of autoxidation exceed the low rate of metHb reduction. A suitable reductant should possess a low rate of autoxidation but a high efficiency of metHb reduction. This can be expressed from k_2app/k_1app (k_1app: the apparent rate constant of metHb reduction in anaerobic condition, k_2app: the apparent rate constant of thiol oxidation by oxygen), and its order is Acy > Cys > GSH > Hcy. Therefore, we selected Hcy as a reductant in HbV to effectively suppress metHb formation.

It is a well-known phenomenon that there is a significant dependence of the rate of metHb formation on the oxygen partial pressure, the rate shows a maximum at a pO_2 around p_{50} of Hb, and superoxide anion generate during metHb formation [24,25]. The coencapsulation of Hcy with Hb resulted in a low rate of metHb formation in HbV (initial rate, 1%/h) in vitro at an oxygen partial pressure (pO_2) of 149 mmHg. The rate increased with decreasing pO_2, showing a maximum (2.2%/h) around $pO_2 = 23$ mmHg, and then decreased to 0%/h at 0 mmHg. At the pO_2 of 149 mmHg, the metHb formation is effectively suppressed by catalase, suggesting the generation of hydrogen peroxide from the autoxidation of Hcy, while at the pO_2

of 23 mmHg the coencapsulation of SOD is necessary because of the generation of superoxide anion from the Hb.

In the intravenous injection of HbV into rats at a 20 vol % overdose, the rate of metHb formation was about twice that measured under physiological conditions in vitro at a pO_2 of 149 mmHg. Though the coencapsulation of active oxygen scavengers such as SOD and catalase suppress metHb formation, the main reason would be due to the ratio of deoxyHb which is oxidized more easily than oxyHb [25].

Another effective method of metHb reduction is the activation of a metHb reduction system when the concentration of metHb becomes high during the course of the blood circulation [26]. One of the possible ways is to add a reductant from the outer aqueous phase of the vesicles when the metHb ratio becomes high; however the bilayer membrane prevents the reductant from contacting and reacting with the inside metHb. The construction of an electron-transfer pathway across the bilayer membrane is necessary, which takes place in natural systems, for instance, the photosynthetic system in plants, the respiratory chain in mitochondria, ion-channels and so on. Model studies of such systems have been carried out using ubiquinones, cytochrome, dyes, and synthetic lipids.

The possibility of metHb reduction was studied using HbVs in which electron mediators and reductants were incorporated [26]. In the case of the addition of water-soluble reductants such as NADH or ascorbic acid (ASH), no reduction was observed because of their impermeability through the bilayer membrane. When ubiquinone was incorporated into the bilayer membrane as an electron mediator [27] under anaerobic condition, the percentage was steeply decreased less than 10% after 20 min. However, the metHb percentage adversely increased under aerobic condition in the case of the quinone/NADH system. Methylene blue (MB) [28] can exist in both aqueous and hydrophobic phases and is used for the treatment of methemoglobinemia [29,30]. When it was employed as an electron mediator instead of ubiquinone, a high reduction rate of metHb and almost complete reduction under anaerobic condition were observed [31]. Under aerobic condition, the MB/NADH or ASH systems showed the decrease in the metHb percentage though the incorporated amounts became high in comparsion with those under anaerobic condition. Therefore, this system might be useful to reduce the metHb in the HbV by simple injection.

Prevention of aggregation of HbV by surface modification

Because the red cell substitutes are expected to be used as a replacement for a large amount of lost red blood cells, the rheological properties of HbV itself and the mixture with blood are important in relation to hemodynamics. Surface modification of the phospholipid vesicles with some natural or synthetic glycolipids [31–34] or polyethyleneglycol (PEG)-conjugated lipids [35–37] is known to improve the dispersion state of the vesicles and prolong the circulation time in vivo for drug delivery systems (DDS). For HbV, the surface was also modified to improve the

dispersion state of the vesicles in the presence of water-soluble polymers or blood components [7,38]. The PEG-modified HbV (PEG-HbV) have been extensively studied in vitro and in vivo, and its oxygen transporting ability has been evaluated [39,40]; we have paid attention to the physicochemical or rheological aspects such as the modification process and the effect of PEG chains on the solution viscosity [41].

Surface modification of HbV with PEG and characteristics of PEG-HbV

In general, PEG-lipid is incorporated into vesicles by mixing with the other lipid components in organic solvents before dispersing them into an aqueous solution. In this case, both inner and outer sides of vesicular membrane are modified. The PEG chains extending from the inner surface should reduce the encapsulation efficiency of Hb, resulting in a low [Hb]/[Lipid] ratio. We used the modification method with PEG-lipid which is added to the outer aqueous phase of the preformed HbV, and have been studying the thermodynamics of PEG-lipid incorporation into vesicles using the isothermal titration calorimetory, and the equilibrium constant using ^{1}H-NMR, to know the optimal molecular structure of PEG-lipid for stable incorporation and effective function.

The PEG-HbV was dispersed into 5 g/dl human serum HSA (HSA) solution, and the resulting PEG-HbV/HSA data are summarized in Table 1. The diameter was controlled to 0.25 ± 0.08 μm. By increasing the weight ratio of Hb to lipid to 1.75, the lipid concentration was reduced to 5.71 g/dl. The high encapsulation efficiency was due to the control of intermolecular interactions during the assembling and sizing procedure of HbV [12]. The lipid composition of the resulting HbV was DPPC/cholesterol/DPPG/α-tocopherol/PEG-DSPE = 5/5/1/0.1/0.014 by molar ratio (PEG-DSPE = 0.13 mol%). More than 99% of the added PEG-DSPE was incorporated onto the outer surface of the HbV. The oxygen affinity, p_{50} was regulated to 32 mmHg by coencapsulating PLP (18.6 mM). The amount of oxygen release was calculated to be 6.2 ml/100 ml, which was close to the 7.0 ml/100 ml of human blood due to the increased oxygen transporting efficiency of the PEG-HbV, 37% compared to that of human red cells, 28%. No leakage of Hb was observed during and after the introduction of PEG-DSPE. The oxygen affinity of PEG-HbV was almost the same as that of HbV within experimental error, indicating that pH change in the inner aqueous phase and the leakage of coencapsulated small molecules such as PLP, Na^+, and Cl^-, were negligibly small. The densities of the PEG-HbV/HSA and HbV/HSA suspensions were almost the same (1.0336 and 1.0335 g/cm^3), and they were smaller than that of human blood (1.0525 g/cm^3), mainly due to the low concentration of HSA solution (5 g/dl), which is lower than the plasma protein concentration (ca 7.5 g/dl). The concentration of HSA of the suspension is expressed as 3.2 g/dl due to 36% of the total volume being HbV particles.

Table 1

Characteristics of HbV and PEG-HbV compared with XLHb abd RBC

Parameters	XLHb[a]	HbV	PEG-HbV	RBC
Diameter (nm)	5	244 ± 70	251 ± 76	8000
Hb (g/dl)	10.0	10.2	10.0	ca. 15
Lipid (g/dl)	–	5.9	5.7	0.2
[Hb]/[Lipid]	–	1.73	1.75	75 (13[c])
PEG-lipid (mol%)	–	–	0.3	–
p_{50} (mmHg)	32	35	35	28
OTE (%)[b]	32	37	38	28
metHb (%)	< 2	< 3	< 3	< 0.5
HbCO (%)	< 2	< 2	< 2	< 5
pH at 37°C	7.4	7.4	7.4	7.4
Osmolality (mOsm)	300	300	300	300
Oncotic pressure (mmHg)	30	20	20	25
Viscosity (cP) at 37°C	1.6	9.4	3.7	4.0

[a]Intramolecular crosslinked hemoglobin with bis(3,5-dibromosalicyl)fumarate (DBBF).
[b]Oxygen transporting efficiency: the difference in oxygen saturation (%) between pO_2 of 40 and 100 mmHg.
[c]Including membrane proteins (ca 1 g/dl) as lipid.

Effect of PEG conjugation on the aggregation of HbV in HSA solution and in blood mixture

The dispersion states of the PEG-HbV/HSA when mixed with human blood were observed by optical microscopy [43]. In the case of PEG-HbV/HSA, only RBCs were confirmed because of the small diameter of PEG-HbV/HSA, and no aggregate of PEG-HbV was confirmed in TEM photograph. In the case of HbV/HSA, on the other hand, aggregates of the HbV were actually confirmed among the RBCs. The RBCs were neither aggregated nor deformed, suggesting that both HbV and PEG-HbV would not interact with RBCs. The aggregates of HbV were actually observed before mixing with blood. It is quite obvious that PEG chains suppress the HbV aggregation.

Figure 3 shows the solution viscosity of the mixtures of HbV/HSA or PEG-HbV/HSA with blood. When the unmodified HbV was dispersed in PBS, the viscosity of the vesicular suspension became 2.6 cP (shear rate = $230\,s^{-1}$), which is lower than that of blood (3.7 cP). However, when dispersed in a 5 g/dl HSA solution to adjust the colloidal osmotic pressure, the HbV/HSA showed 8 cP viscosity (shear rate = $358\,s^{-1}$), which was substantially higher than that of blood [42]. The HbV/HSA shows a non-Newtonian flow typical for particle suspensions, while PEG-HbV/HSA shows a Newtonian flow and was almost the same as that of human blood at any shear rates. These results indicate that the unmodified HbV aggregates due to the molecular interaction of HSA with the vesicular surface and increase viscosity, while the surface modification of the HbV with PEG chains suppresses HbV aggregation and provides a low viscosity almost the same as that of human blood.

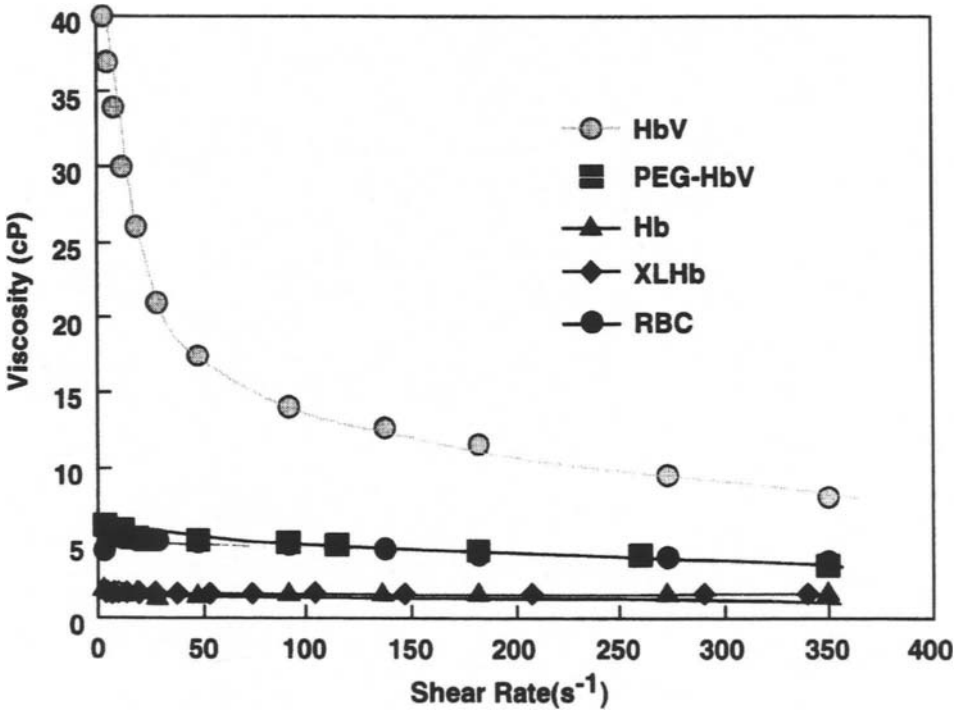

Fig. 3. The profiles of the shear rate dependent solution viscosity of oxygen carriers. The profiles of HbV and PEG-HbV are compared with those of Hb and RBC.

The permeabilities of the PEG-HbV/HSA, HbV/HSA and human blood through isopore membrane filters were studied as a model of the blood flow through capillaries [42]. This method is conventionally used for the RBC deformability measurement. The capillary diameter is usually 5–10 μm. It is generally known that RBC can penetrate through the 5 μm pore size of the membrane filter. A biconcave-shaped RBC with a diameter of 8 μm deforms to a parachute-like configuration and penetrates through the capillaries of ca 5 μm diameter. However, with decreasing pore size of the membranes from 5 to 2 μm, the flow rate decreased for all suspensions, especially for human blood even though the applied pressure is about twice (220 mmHg) that of normal blood pressure. When the pore diameter is 3 μm, blood could hardly penetrate, though both the unmodified HbV/HSA and PEG-HbV/HSA showed high penetration. Due to the small size of HbV (250 nm), both of the suspensions promptly penetrate through the membrane filters with pores of sizes down to 0.4 μm without changes of vesicular size or Hb leakage, and especially the PEG-HbV/HSA, which is not aggregated, penetrates faster than the unmodified HbV/HSA. The HbV/HSA showed aggregation in the optical microscopy; nevertheless, the aggregates dissociated at higher shear rates and penetrated more promptly

than expected. Both the HbV and PEG-HbV can penetrate through sterilizable filters of 0.22 μm in pore size for preparation.

In vivo evaluation of the efficacy of HbV

Ninety percent exchange transfusion tests

Ninety-percent of the estimated circulatory volume of rats was exchanged with 5 g/dl human serum albumin (HSA group, $n = 6$), ratRBC/HSA ($n = 6$) or PEG-HbV/HSA ($n = 6$) at 2 ml withdrawal (via the common carotid artery)/infusion (via the right artrium) cycles at a rate of 2 ml/min [43]. A needle-type polarographic oxygen electrode was placed in the cortex of the left kidney for the continuous measurements of renal cortical tissue oxygen tension. Blood samples for arterial blood gas analyses were taken on the first withdrawal as baseline values, and thereafter at exchange ratio of 10, 40, 60, 70, 80, 90% and at 30 min after the completion of the exchange transfusion. The results were summarized in Fig. 4. All data are shown as percentage of the baseline values and are expressed as mean ± standard deviation (S.D.).

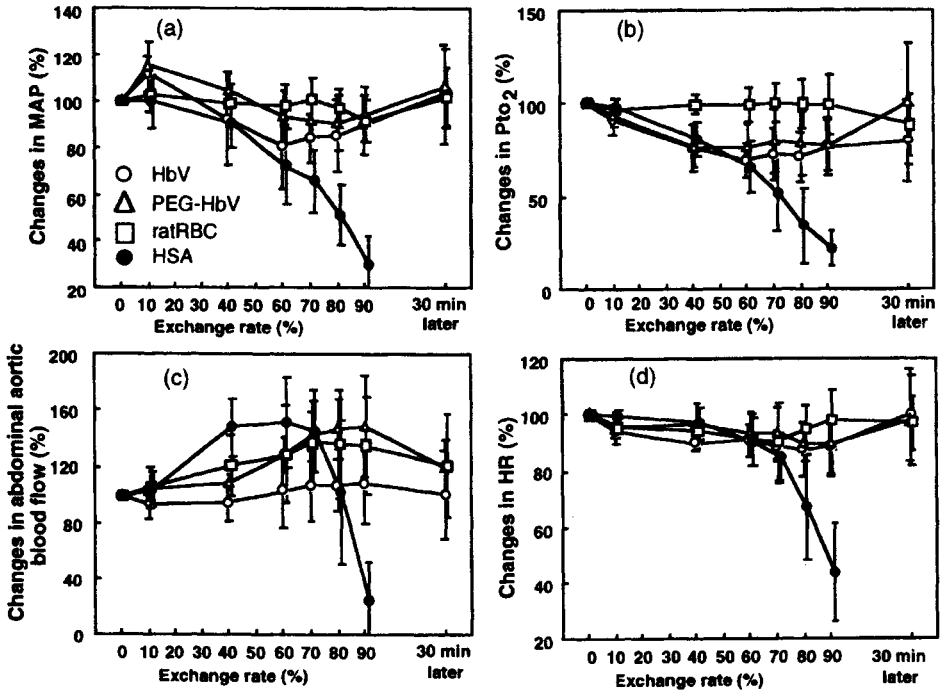

Fig. 4. Changes in (a) mean arterial pressure (MAP); (b) renal cortical tissue oxygen tension (p_tO_2), (c) abdominal aortic blood flow, and (c) heart rate (HR) during the exchange transfusion tests. They are expressed as percentages of the baseline values.

During the exchange transfusion, the hematocrit of blood in both the HbV/HSA and PEG-HbV/HSA groups decreased from about 50 to 5%. This indicates that almost 90% exchange transfusion with HbV/HSA was actually performed. After the hematcrit measurement, the HbV layer was confirmed on the RBC layer in the glass capillary; while PEG-HbV was dispersed in the supernatant not forming a layer. The changes in mean arterial pressure (MAP) showed a slight transient increase in the HbV/HSA and PEG-HbV/HSA groups, then decreased to 90% of the baseline values, and sustained at that level throughout the experiment. There was no significant difference in MAP between HbV/HSA and PEG-HbV/HSA groups. On the other hand, MAP in the HSA group declined to 66.0%, which was significantly lower than the other groups, and from there it continued to decline to zero within 20 min after the completion of the exchange transfusion, meaning death. The renal cortical oxygen tension (p_tO_2) for the HbV/HSA and PEG-HbV/HSA groups decreased to approximately 70% of the baseline values at 90% exchange, and the values were significantly lower than that of ratRBC/HSA group, though they were significantly higher than that of HSA group.

Aortic blood flow in the HSA group increased with exchange ratio and reached to 160% of the baseline up to a 40% exchange ratio, and then showed a significant decrease to 25% at an exchange ratio of 90%. Exchange transfusion with 5 g/dl HSA, a non-oxygen carrying colloid induces isovolemic anemia. At 40% exchange, in the HSA group, cardiac output increased in order to sustain oxygen delivery, which was depicted as an increase in aortic blood flow. However, when the exchange rate exceeded 70%, this compensation could no longer sufficiently function because of the impairment in cardiac function caused by decreased oxygen delivery to the cardiac muscles themselves. On the other hand, PEG-HbV/HSA and ratRBC/HSA groups showed 135–150% of baseline, while the HbV/HSA group sustained a baseline value. Regarding the parameters of heart rate, pH, and base excess, no significant differences were confirmed among those three groups, PEG-HbV/HSA, HbV/HSA, and ratRBC/HSA and the baseline values were sustained. In the HbV/HSA group, oxygen delivery tended to be lower and oxygen consumption tended to be higher those of the PEG-HbV/HSA and ratRBC/HSA groups without significant differences.

In conclusion, hemodynamic and blood gas parameters as well as tissue oxygen tension measurements were well sustained in the HbV/HSA and PEG-HbV/HSA groups during and after the exchange transfusion. This indicates that oxygen transport was satisfactorily maintained by HbV in these animals. The PEG-HbV/HSA group showed higher aortic blood flow than HbV/HSA and ratRBC/HSA groups. This was considered to be the result of the lower viscosity of the PEG-HbV/HSA.

Rabbit shock model

In a state of shock, intestinal ischemia and its impaired barrier function may result in the precipitation of multiple organ failure. When solutions of oxygen carriers are infused in states of ischemia for resuscitation, the differences in the oxygen transporting capability of the solutions would cause the differences in the recovery of intestinal

ischemia. In order to evaluate the oxygen transporting capability of HbV which encapsulates concentrated hemoglobin, a rabbit shock model was used and oxygen transport to the small intestine was measured.

A 2 mm ultrasonic flow probe was placed around the superior mesenteric artery for a measurement of arterial flow. The catheter was advanced 5–10 cm proximally until the tip was located in the superior mesentric vein for sampling of venous blood. A sigmoid tonomitor was positioned in the duodenum 2–3 cm from the pylorus for a measurement of intestinal mucosal pH. A needle type polarographic oxygen electrode was inserted in the submucosa of the small intestine for continuous intestinal tissue oxygen tension measurements.

Shock was induced by withdrawal of 40% of the estimated circulatory blood volume followed by isovolemic infusion of fluids; PEG-HbV dispersed in 5 g/dl HSA (PEG-HbV/HSA), 5 g/dl HSA (HSA), and washed rabbit red cells dispersed in 5 g/dl HSA(RBC/HSA). The hemoglobin concentration of those samples was adjusted to 10 g/dl. The procedure was repeated twice.

From the results concerning on MAP, HR, arterial blood O_2 tension (p_aO_2), and blood flow in the superior mesenteric artery, there were no significant differences between groups. In other words, volume resuscitation with HSA alone was sufficient to sustain these parameters. On the other hand, systemic BE, intestinal mucosal pH, and intestinal tissue O_2 tension (p_tO_2) were sustained significantly higher in the PEG-HbV/HSA and RBC/HSA groups compared to the HSA group. Concerning venous blood O_2 tension (p_vO_2), the higher value in the HSA group probably resulted from shunting of the tissues due to the collapse of peripheral circulation. The small intestine is one of the most vulnerable organs in shock and from these results we can assume that intestinal perfusion was more optimal in the PEG-HbV/HSA and RBC/HSA groups due to their similar oxygen transporting capabilities.

Conclusions

Concentrated hemoglobin could be encapsulated in the phospholipid vesicle by the control of molecular interaction between hemoglobin and phospholipids. The oxygen affinity and the rate of methemoglobin formation are controlled by the coencapsulation of allosteric effectors, reductants, and by the solution pH. Construction of more sophisticated artificial methemoglobin reduction systems would be possible if the kinetics of autoxidation including the generation of active oxygens and the reduction of methemoglobin through the bilayer membrane were studied. The increase in the viscosity of the mixture of blood and HbV was due to the aggregation of vesicles. It was suppressed by the surface modification with PEG chains. The effect of the PEG modification of the HbV were also observed in vivo experiments as the increased blood flow, and the resulting stable gas parameters. The PEG-HbV transported oxygen almost as well as red blood cells and can be considered as a promising candidate for an artificial oxygen carrier.

Acknowledgments

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 15

Microvascular Responses to Hemodilution with Hb-Vesicles: Importance of Resistance Arteries and Mechanisms of Vasoconstriction

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Introduction

Phospholipid vesicles encapsulating concentrated hemoglobin (Hb vesicles, HbV) have the potential of becoming industrially produced red cell substitutes. They most closely reproduce the characteristics of natural blood including the red cell membrane function of physically preventing direct contact of Hb with the cellular components of circulation [1–3]. The desirability of this barrier function is evident in considering the side effects found in the use of acellular Hb solutions such as chemically-modified Hb and recombinant Hb which are now in clinical trials.

The principal systemic side effect consistently reported in the administration of a red cell substitute based on Hb solutions is a pressor response [4,5]. This has been widely regarded to be due to the nitric oxide (NO) scavenging effect of Hb, caused by the intrinsic high affinity of NO to Hb, a process presumed to evoke vasoconstriction [6]. Even though the pressor effect has been proposed to be beneficial as a remedy for hypotension in endotoxin shock, vasoconstriction is deleterious to the downstream microvascular function and tissue oxygenation. Conversely, it has been confirmed that NO-related vasoconstriction by the liposome-encapsulated hemoglobin (Hb-vesicles, HbV) does not occur in an ex vivo experiment using a rabbit aortic strips [7]. Direct microcirculatory observation, using a conscious hamster fitted with a dorsal skin window, has shown that arterioles (diameter, less than ca. 50 μm) do not constrict [8]. Since local biochemical events interact with systemic regulation, the understanding of phenomena such as Hb induced vasoconstriction requires the combined microscopic and systemic analysis of vascular function [9].

The presence of red cell substitutes that utilize Hb as an oxygen carrier induces vasoconstriction by variety mechanisms in addition to the NO–Hb reaction, all of which affect the "resistance vessels" that regulate peripheral blood flow [10]. At first this chapter summarizes the non-invasive technique to observe the microvascular perfusion and the responses to the hemodilution with HbV. Then, we discuss

proposed mechanisms which may cause vasoconstriction and reduced downstream blood flow, and discusses applications to the design of red cell substitutes to improve microcirculation and tissue oxygenation.

Microhemodynamic measurements and hemoglobin vesicles

Method to observe microhemodynamics and tissue oxygenation

Types of the animal preparations for microvascular observation which are often used are, sartorius, cheek pouch, cremaster, conjunctiva, cremaster, mesentery, pia mater, skeletal muscle, etc., or in special case bat wings. An ideal preparation should have the following properties: no anesthesia, no trauma, immobility, transparency, accessibility and undisturbed environment of the tissue (no irrigation, plastic films, air, etc.) [11,12]. Hamster dorsal skin fold window chamber preparation is currently used in our experiment of hemodilution with red cell substitutes because of no need of anesthesia during observation, less trauma, enough transparency, and long term potency for observation [8,9]. A cover glass (diameter, 12 mm) was surgically installed on the exposed skin allowing intravital observation of the microvasculature and tissues as shown in Fig. 1. Polyethylene catheters were implanted in the jugular vein

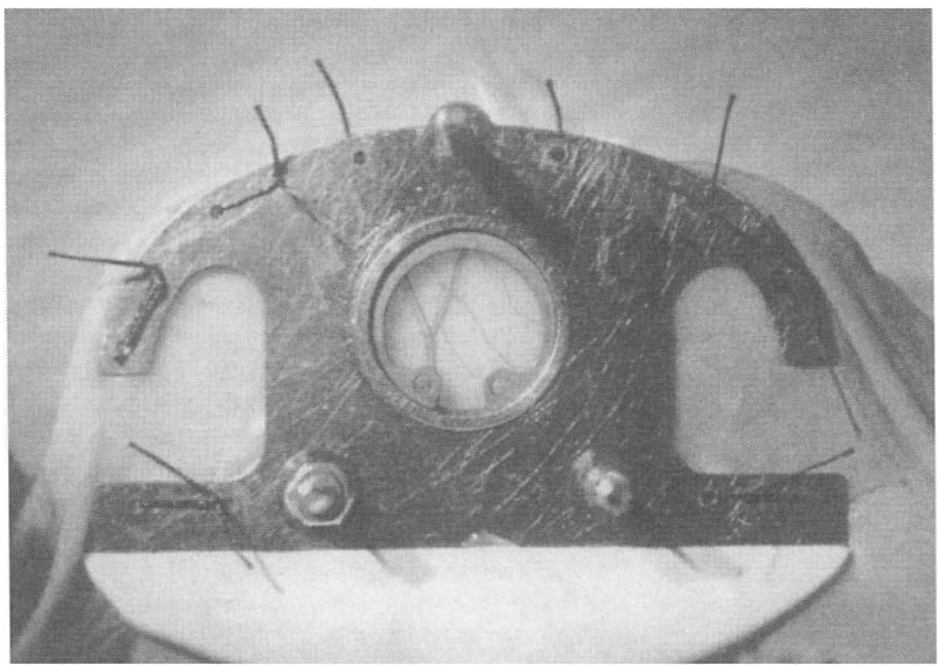

Fig. 1. Dorsal skinfold chamber in a Syrian golden hamster used to visualize microvessels in the subcutaneous tissue.

and the carotid artery. They were passed subcutaneously from the ventral to the dorsal side of the neck and exteriorized through the skin at the base of the chamber. During the measurement, the animals were placed in a porous plastic tube from which the window chamber protruded to minimize animal movement without impeding respiration.

Mean arterial pressure (MAP), heart rate, blood gas, and hematocrit were measured from arterial line. Microvessels in the subcutaneous tissue and the skeletal skin muscle were observed with an inverted microscope with a trans-illumination technique (Fig. 2). Microvascular diameter was measured with an image-shearing

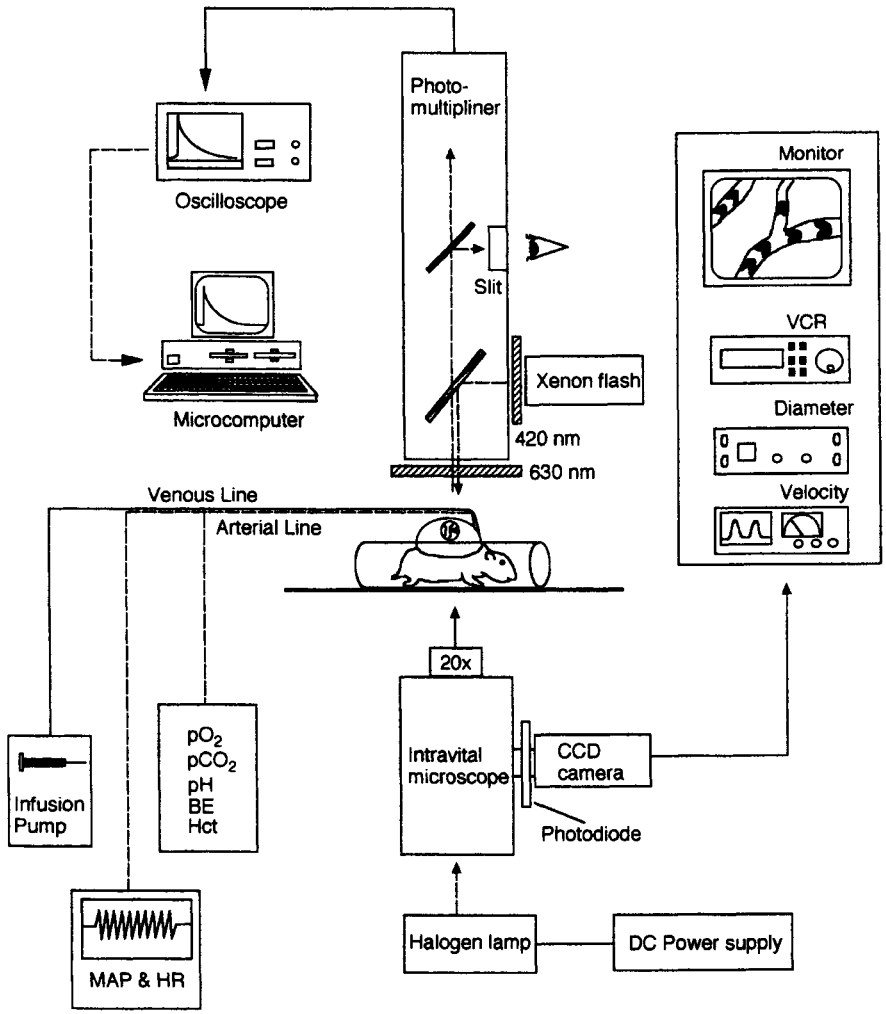

Fig. 2. Schematic diagram of system for the measurement of hemodynamic parameters and oxygen tensions in microcirculation.

system (Digital Video Image Shearing Monitor 908, I.P.M. Inc.), while RBC velocity was analyzed with photodiodes and the cross-correlation technique (Velocity Tracker Mod-102 B, I.P.M. Inc.) [13,14]. Blood flow rates (Q) were calculated using the RBC velocity and the diameter. Functional capillary density was analyzed on-line by counting the number of capillaries stemming from one A_3 arteriole.

Subcutaneous microvascular and interstitial pO_2 values were determined with the O_2 dependent quenching of phosphorescence emitted by bovine serum albumin bound paradium-*meso*-tetra(4-carboxyphenyl)porphyrin complexes after pulsed light excitation [15]. The method allows non-invasive assessment of intravascular pO_2 and determination of interstitial oxygenation, since intravascularly injected porphyrin-albumin extravasate into the interstitium over time. Phosphorescence was excited by xenon strobe arc, while pO_2 measuring sites were microscopically vignetted by an adjustable slit, and the signals were captured by a photomultiplier. One hundred and twenty eight decay curves were averaged, visualized and the pO_2 was obtained with computer fitting to a single exponential, using the Stern-Volmer equation.

Hemodilution with hemoglobin vesicles modified with polyethyleneglycol

Using the intravital microscopic method of hamsters mentioned above, the function of HbV as a blood replacement was tested during severe hemodilution where 80% of the red blood cell mass was substituted with suspensions of the vesicles in 5% human serum albumin (HSA) solution [8]. The characteristics of HbV were, diameter = 258 ± 57 nm, oxygen affinity = 31 mmHg, and [Hb] = 10 g/dl. Vesicles were tested with membranes that were unmodified (HbV/HSA) or conjugated with polyethyleneglycol (PEG) on the vesicular surface (PEG-HbV/HSA). The viscosity of 10 g/dl HbV/HSA is 8 cP at 358 s^{-1} due to the intervesicular aggregation, while that of 10 g/dl PEG-HbV/HSA is 3.5 cP since PEG chains inhibit aggregation. Both materials yielded normal mean arterial pressure, heart rate, and blood gas parameters at all levels of exchange that could not be achieved with HSA alone. Subcutaneous microvascular studies showed that PEG-HbV/HSA significantly improved microhemodynamic conditions (flow rate, functional capillary density, vessel diameter, oxygen tension) relative to unmodified HbV/HSA. PEG-HbV was homogeneously distributed in the plasma phase, while aggregates of the unmodified HbV were clearly observed in capillaries and venules where the flow rate were low (Fig. 3). Even though it is confirmed in vitro that the aggregates dissociate reversibly at higher shear rates, it is unlikely that they will dissociate in vessels where the flow rate or shear rate is low. Aggregation and decreased flow rate may constitute a vicious circle that reinforces negative effects on blood flow. Thus PEG reduced vesicular aggregation and viscosity improving microvascular perfusion relative to the unmodified type.

In this experiment, it has been clarified that arterioles (diameter, less than ca. 50 μm) do not constrict. However, we speculated that the upstream vessels such as small arteries and thoracodorsal arteries, not visible in the preparation, were also

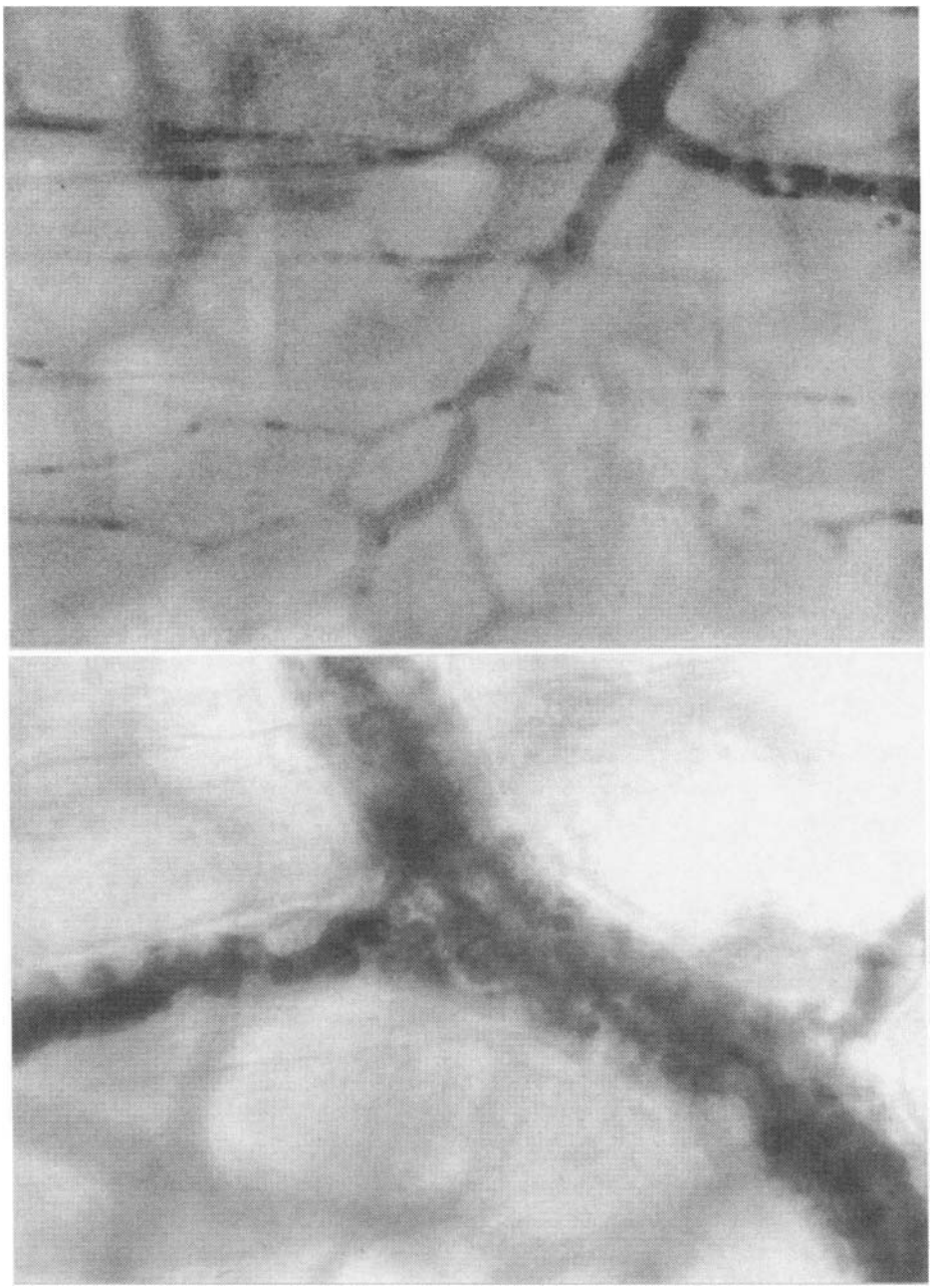

Fig. 3. Micrographs of microvasculature after 80% exchange with PEG-HbV/HSA and unmodified HbV/HSA. (Top) Microvasculature are blackened owing to the homogeneous distribution of PEG-HbV in the plasma phase. (Bottom) The aggregates of HbV form blocks in collecting venules.

important to observe to elucidate the mechanism of controlling downstream microcirculation.

Importance of arterioles or small arteries (resistance vessels) for the microcirculation

Approximately a half of the total blood pressure drop across the microvasculature occurs in the small arteries and arterioles, termed "resistance vessels" in vascular networks such as the mesentery, pia mater, skeletal muscle, cremaster, and cheek pouch as shown by Davis et al. [16] (Fig. 4). Vascular resistance is dominated by adrenergic constriction and intrinsic smooth muscle tone of metabolic, myogenic and autacoid origin [17–19]. However, most of the microcirculatory studies after infusion of red cell substitutes did not cover these vessels because of the difficulty in access especially in unanesthetized in situ condition [8,15,20].

Non-invasive observation of resistance vessels

The behavior of the microvasculature and corresponding larger feeding arteries and capacitance veins was analyzed for the first time in the conscious hamster dorsal skinfold window preparation by shifting the position of the window to include the vessels [21]. These vessels correspond to circumflex scapula in humans. The diameter of the artery is of the order of 150 μm and the vein diameter is in the range of 300–400 μm, and they run in parallel together with the major nerves of this tissue. These vessels exhibited the most significant responses during hemodilution with human serum albumin (HSA), both reducing their diameter to 70% of control, while the

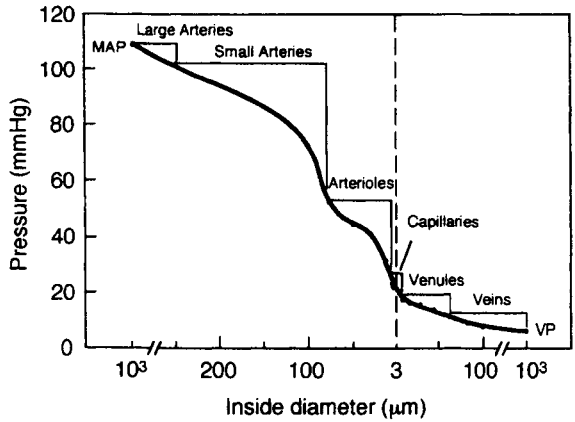

Fig. 4. Pressure drop across the vascular system in the hamster cheek pouch. MAP, mean arterial pressure; VP, venous pressure (cited from Davis et al., Am. J. Physiol., 250, H291, 1986).

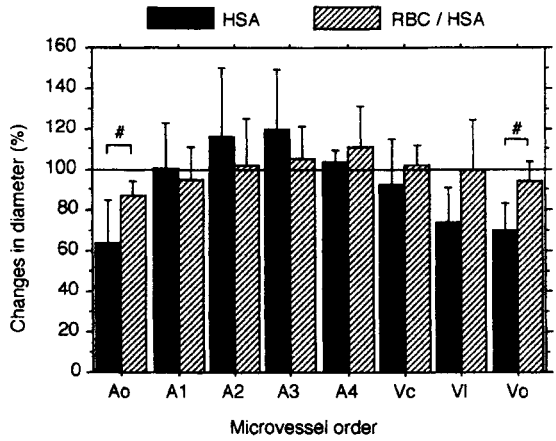

Fig. 5. Diameter changes of microvasculature in conscious hamster dorsal skinfold preparation after 80% hemodilution with 8% human serum albumin (HSA) and washed RBCs suspended in HSA (RBC/HSA) with hemoglobin concentration of 10%. Baseline diameters (in μm); Ao (150); A_1 (50); A_2 (20); A_3 (10); A_4 (8); Vc (30); V_1 (80); Vo (300). Values are mean ±stanadard deviation. # significant difference ($p < 0.05$).

smaller arterioles usually investigated in microvascular studies, with diameters in the range of 50–70 μm did not exhibit any significant change (Fig. 5). The significant constriction of the feeding arteries may help to maintain blood pressure while constriction of the capacitance veins may contribute to redistribute blood from skin to vital organs.

The small arterioles showed a nonsignificant tendency to dilate, however, blood flow was not affected as a consequence of the upstream arterial constriction, and the microvasculature became hypoxic. Hemodilution with washed RBC suspended in HSA did not show such vasoconstriction, and blood flow was maintained with higher microvascular oxygen tensions. The two reperfusion media differ physically both in terms oxygen carrying capacity and viscosity, the latter probably inducing changes in shear stress at the vascular wall. Our recent observation showed that immediately after the 10 vol% toploading of acellular α,α-crosslinked Hb (XLHb, [Hb] = 5 g/dL) into hamsters, they started to show hypertension (+30 mmHg) with simultaneous vasoconstriction of A_0 (−23%) as shown in Fig. 6, but not with A_1. On the other hand, cellular HbV group (data not shown here), as well as HSA group did not show such dramatic changes. The difference may be mainly explained with the nitric oxide scavenging effect of acellular XLHb, which can easily approach to the smooth muscle of the artery and binds NO, while the larger HbV particles with diameter of about 200–250 nm can not come close to the smooth muscle. Nakai et al. has shown that the molecular size of Hb products influences the permeability of endothelial cell layer and accessibility to the smooth muscle [Chapter 20, ref 7]. These tests indicate the importance of observing the resistance vessels that bridge the systemic vasculature and the microvasculature in evaluating efficacy and side effects of red cell substitutes.

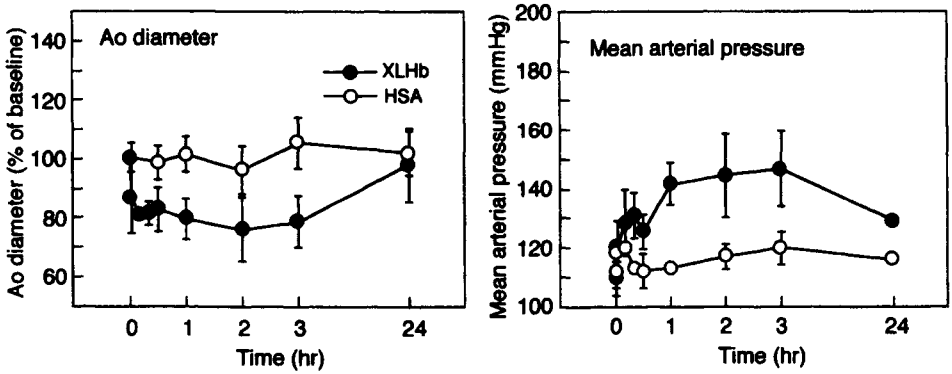

Fig. 6. Time course of A_0 diameter and mean arterial pressure after 10% toploading of intramolecularly cross-linked Hb (XLHb, 5 g/dL) and human serum albumin (HSA, 5 g/dL).

Oxygen dependent vasoconstriction

Blood flow toward many organs appear to be closely regulated such that the tissue receives an adequate supply of O_2 to satisfy the requirements of oxidative metabolism under a wide variety of circumstances. Arterial constriction and/or the decreased functional capillary density in conditions of increased pO_2 have been observed in several studies [22–26], where the vasoconstriction has been attributed to the lowered production of endothelium-derived prostacyclin. Recently, the cellular O_2 sensor was identified as a cytochrome P450 enzyme that catalyzes the arachidonic cascade to produce constrictor hydroxyarachidonic acid [27]. Thus, several blood flow regulatory mechanisms appear to prevent an overabundance of O_2 delivery to tissues.

Transfer of O_2 from acellular Hb or cellular HbV to the vascular wall is faster than from RBCs as shown by stopped flow in vitro analysis (k_{off}; Hb : $80\,s^{-1}$, HbV : $30\,s^{-1}$; RBC : $4\,s^{-1}$) [28]. This is due to morphological differences arising from the diffusion of the O_2 molecule form the viscous and concentrated Hb solution in RBCs and the plasma barrier present between RBCs and the vascular wall [29], a situation markedly different from the rapid release of O_2 from the homogeneous dispersion of Hb in plasma. Although HbVs have a cell-like structure, they release O_2 at a faster rate than RBCs due to their smaller size (250 nm) and consequent greater dispersion homogeneity. Although the faster release of O_2 could be advantageous, it is also possible that it may lead to the autoregulatory vasoconstriction [9,30,31].

Vasoconstriction has also been attributed to the O_2 binding-dissociation equilibrium of the O_2 dissociation curve of Hb rather than the rate of O_2 release. Transmembrane introduction into RBCs of a synthetic allosteric effector such as inositol hexaphosphate reduces the O_2 affinity of Hb, which increases arterio-venous O_2 saturation difference and increases the amount of O_2 released from RBCs. Reduction of cardiac output with maintenance of constant systemic pressure was found after infusion of an allosteric effector, or the introduction of blood with

right-shifted RBCs, indicating the increase of peripheral resistance by arterial vasoconstriction in response to overabundant O_2 supply [32–34]. The pressor reaction was not confirmed and not detrimental to tissue oxygen tension with the use of modified RBCs.

Our recent study of conscious hamster dorsal skinfold microcirculation during hemodilution with a low O_2 affinity HbV (p_{50} = 53 mmHg) showed that microvascular perfusion was reduced even though arterioles (diameter < 50 μm) slightly dilated. However, upstream small arteries (diameter, ca. 150 μm) constricted significantly. Comparing the shape of O_2 dissociation curve of HbVs with that of hamster RBCs, shows that the slopes at pO_2 = 60 mmHg is steeper for HbVs than for RBCs. Since this oxygen partial pressure was found in the same arteriolar vessels for both types of solutions, it is likely that HbVs release a larger amount of O_2 in these vessels than the RBCs, which may induce vasoconstriction. Conversely, higher O_2 affinity HbVs (p_{50} = 16 mmHg) had a tendency to cause higher microvascular perfusion.

These findings suggest an important concept in design of a red cell substitute: in normal conditions the O_2 dissociation curve of RBCs is regulated not to release a lot of O_2 before entering the microcirculation. Furthermore, infusion of red cell substitutes with a right shifted O_2 dissociation curve does not necessarily enhance microvascular perfusion. These considerations lead to the hypothesis that there may be an optimal oxygen affinity of red cell substitutes for each clinical setting.

Other mechanisms of vasoconstriction caused by red cell substitutes

Adrenoreceptors and endothelin

αα-crosslinked Hb (XLHb) has been reported to induce vasoconstriction and resulting hypertension by NO-trapping and also in part by adrenergic responses or endothelin production [35–37]. There is evidence that the pressor effect of XLHb originates in the peripheral vascular system and is not mediated by the central nervous system, since XLHb potentiates the pressor responses of norepinephrine, phenylephrine and clonidine, indicating increased sensitivity of peripheral vascular α-adrenoreceptors. The pressor effect of XLHb could be reversed by administration of prazosin and yohimbine, an α_1- and α_2-adrenoreceptor antagonists, respectively. Phosphoramidon, an inhibitor of proendothelin conversion to endothelin also attenuates the pressor effect of XLHb.

Although the location of these receptors and their mechanism of action is not well understood, resistance arteries are known to be rich in adrenoreceptors [38] which regulate the downstream microcirculation. Understanding of these mechanisms will provide the opportunity of controlling or taking advantage of the pressor effect.

Shear stress on the vascular wall and EDRF

It has been reported that increased blood flow acts as an important signal for the coordination of vasodilation along the arterial tree. The ability of small and large

vessels to dilate in response to elevated blood flow has been demonstrated [39]. This flow-induced dilation is endothelium-dependent and primarily mediated by nitric oxide, and in some cases by prostacyclin [40].

It has been proposed that the viscous drag exerted on the endothelial cells by the flowing blood, wall shear stress, triggers flow induced dilation. Wall shear stress is expressed as $8V_m\eta/D$, where, V_m is the mean red blood cell velocity, η is the viscosity, and D is the vessel diameter. Hemodilution with plasma expanders inevitably reduces blood viscosity leading to lowered shear stress. To maintain wall shear stress and diameter, viscosity should be increased. Studies from our and another groups tend to confirm the beneficial effect of perfusion with high molecular weight dextran solutions as a viscous plasma expander [41,42], therefore acellular Hb solutions, which have a lower viscosity than blood, may induce vasoconstriction due to lower shear stress (See the corresponding Chapter 10 in this text, [43].

Control of microvascular function by ATP release from RBCs

Human RBCs have been shown to release adenosine 5′-triphosphate (ATP) in response to the combined effect of hypoxia and hypercapnia. ATP is known to bind to receptors localized on the luminal surface of the endothelium in the peripheral circulation [44]. The binding of ATP to these receptors has been shown to induce the production of NO and prostacyclin, both of which are strong vasodilators of the microvasculature.

Ellsworth et al. [45] demonstrated that ATP production is enhanced at low pO_2 and pH, indicating that these factors associated with an impaired O_2 supply increases the release of ATP from RBCs. Intraluminal loading of ATP to small arteries with diameter 140–60 μm, namely resistance vessels, showed increased diameter and blood supply.

Most of the glycolytic enzymatic system is removed from Hb-related red cell substitutes during Hb extraction from RBCs and purification, preventing the production of ATP and vasodilatory activity. The process of the Terumo Co. (Tokyo, Japan) purifies Hb without removing the enzymatic systems [46], in such a fashion that the product maintains metHb reducing enzymatic activity in liposome-encapsulated Hb, which would facilitate the production of ATP and the maintenance of its vasidilatory activity.

Active oxygen species and vasoactivity

Endothelial cells contain xanthine dehydrogenase/oxidase leading to the production of active oxygen species [47], which are reduced by the cell's superoxide dismutase, catalase, etc. Low intracellular levels of active oxygen species stimulate cyclooxygenase. Superoxide radicals can induce vasodilation in several tissues which is in part mediated by the release of prostacyclin from endothelial cells. In normal condition, a balance exists between the production of prostaglandin (dilator), and thromboxane A_2 (constrictor), and between NO and active oxygen species that allows for the

maintenance of vascular tone. However, increased intracellular concentration of active oxygen species inactivates NO, and inhibits the production of prostacyclin in endothelial cells by inhibiting the arachidonic acid cascade. Higher levels of active oxygen species result in the destruction of the enzymes, with the exception of thromboxane A_2 synthetase that is resistant to inhibition and destruction by free radicals. The net effect of these interactions is vasoconstriction [48,49]. Moreover, active oxygen species generates peroxides, which will also deteriorate the enzyme activity.

A common condition to Hb-based red cell substitutes is the relatively rapid metHb formation in the circulation. It is well known that HbO_2 dissociates into metHb and O_2^-. Although the direct relationship between vasoconstriction and O_2^- generation from red cell substitutes has not been well established, suppression of metHb formation may be important not only to maintain the function but also to reduce the generation of superoxide which may act as a vasoconstrictor. In RBCs metHb reduction system includes cytochrome b_5, NADPH-flavin, direct reduction by glutathione, and ascorbic acid, and scavengers for active oxygen species such as superoxide dismutase for O_2^- and catalase for H_2O_2. Preservation of the native enzyme activities is one method of reducing generation of metHb and active oxygen species [46]. However, during the purification of Hb especially at the heat sterilization, the enzymes are completely removed. In the case of acellular Hb, direct conjugation of catalase and superoxide dismutase has been studied to suppresses metHb formation [50]. For cellular HbV, preservation of native enzymatic systems [46] or encapsulation of a certain amount of reductants and the enzymes have been proposed as a method for suppressing metHb formation [51], which may be effective to reduce active oxygen species generation and vasoactivity.

Complement activation and vasoactivity

Anaphylatoxins are pharmacologically active peptides formed in blood during enzymatic activation of the complement system by the presence of foreign elements or inflammation. C3a, a peptide derived from the third component of complement, stimulates smooth muscle contraction, releases histamine from mast cells and increases vascular permeability. Topical applications of C3a and histamine induce vasoconstriction of feeding arterioles [52], and the anaphylatoxin C5a stimulates platelet activity and arachidonic acid cascade releasing thromboxane A_2, which is a strong vasoconstrictor.

Fluosol-DA and some liposome products have been reported to show anaphylactic reactions with thrombocytopenia and increase blood thromboxane A2 concentration [53,54], though vasoconstriction was not studied. Infusion of prednisolone, indomethacin, or complement receptors is one method to prevent complement activation. Surface modification of liposome-encapsulated hemoglobin with polyethyleneglycol (PEG) is another way [8,55] to prevent access of plasma proteins including complement. The effectiveness of PEG in improving blood rheology was reported by the

groups at Waseda and Terumo, and effectiveness in the reduction of complement activation was reported by the Naval Research group at the 7th ISBS.

Summary

Analysis of the microcirculation is important to evaluate the efficacy of red cell substitutes because this is the site of oxygen exchange. PEG-modified HbV have been developed with stable and uniform characteristics that show improved microvascular responses to severe hemodilution compared with unmodified HbV and albumin alone. There have been many vasoconstrictive factors reported so far in the presence of red cell substitutes, which cannot be explained solely on the basis of nitric oxide-related reactions. Resistance vessels appear to be crucial for the regulation of microvascular flow, and the study of these vessels may be important to improve the efficacy of red cell substitutes.

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 16

Hemoglobin-based Blood Substitutes and Mechanisms of Toxicity*

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Summary

Vascular endothelium, a source of a number of oxidants, has emerged as the primary target of hemoglobin-based toxicity, due to its proximity and direct contact with the circulating protein. Reaction of hemoglobin with endothelial signaling molecule, nitric oxide ($NO^{\bullet}$), results in the diversion of $NO^{\bullet}$ away from the smooth muscle target enzymes, leading to the loss of $NO^{\bullet}$ dependent responses such as vasodilatation. The ensuing oxidative reactions of hemoglobin with oxidants such as peroxynitrite ($ONOO^{-}$), the reaction byproduct of nitric oxide with superoxide ($O_2^{\bullet -}$) hydrogen peroxide or lipid peroxide may contribute to the level and extent of tissue damage. In this chapter, an overview of hemoglobin reactions with nitric oxide/peroxynitrite and superoxide/peroxide will be presented together with the emerging understanding of the dynamics that govern hemoglobin's redox reactions with these oxidants and some of the protective strategies under development to combat these reactions.

Oxidants of the vascular system

Vascular endothelium produces, as part of its normal function, a number of oxidants and free radicals that also present a potential route to pathophysiological events. Recent research has shown that nitric oxide ($NO^{\bullet}$) and superoxide ($O_2^{\bullet -}$) play a crucial role in the control of vascular function [1]. $NO^{\bullet}$ a radical molecule, with an unpaired electron, is produced by the vascular endothelial system (Fig. 1(a)). The enzyme nitric oxide synthase (NOS) uses L-arginine and a number of other cofactors to synthesize $NO^{\bullet}$ and L-citrulline. Constitutive as well as inducible forms of NOS have been described. The constitutive forms are calcium dependent enzymes, whereas, the induction of NOS can occur in response to variety of stimuli [2]. Once formed,

* The opinions and assertions contained herein are the scientific views of the author and are not to be construed as policy of the United States Food and Drug Administration.

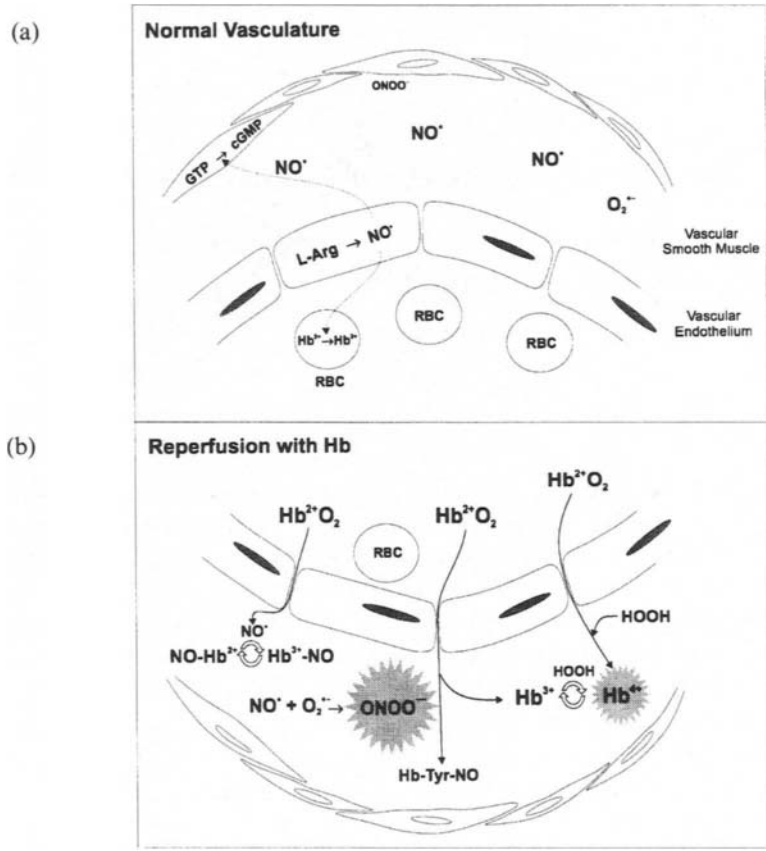

Fig. 1. Potential oxidative reaction pathways of cell-free hemoglobin in the vasculature.

(a) Normal vasculature; Formation of nitric oxide ($NO^{\bullet}$) is catalyzed by the enzyme NO synthase (eNOS) from L-arginine and several other cofactors. The principal action of $NO^{\bullet}$ is to bind to and activate smooth muscle enzyme, guanylate cyclase. This leads to the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) which is required for muscle relaxation. When $NO^{\bullet}$ diffuses into the red cells on the luminal side, it will react with oxyhemoglobin ($Hb^{2+}O_2$) to form nitrate and methemoglobin ($HbFe^{3+}$). The concentration of $HbFe^{3+}$ is kept low by the red cell reducing enzyme systems. The concentration of superoxide ($O_2^{\bullet -}$) in the vasculature is kept at a remarkably low level by a high concentration of superoxide dismutase (SOD). A balance between $NO^{\bullet}/O_2^{\bullet -}$ is therefore maintained under normal physiological conditions and a reaction between the two to form $ONOO^-$ is thus limited.

(b) Reperfusion with cell-free hemoglobin; An extremely rapid initial reaction between $Hb^{2+}O_2$ and $NO^{\bullet}$ results in the formation of $HbFe^{3+}$ and possibly other oxidation products. The reduction in the normal levels of $NO^{\bullet}$ drives the formation of $ONOO^-$. Once its formed $ONOO^-$ can directly and rapidly oxidize the heme to its ferric form ($HbFe^{3+}$) and modify the globin (tyrosine nitration). Hemoglobin and its oxidation products (methemoglobin ($HbFe^{3+}$), ferryl hemoglobin ($HbFe^{4+}$), heme, heme-breakdown products and free iron) residing in the vascular pool may trigger and sustain a series of oxidative reactions ultimately leading to endothelial injury.

nitric oxide in one half life ($\sim$ 1 s) can diffuse over several cell lengths, controlling smooth muscle cell relaxation through the direct activation of the target enzyme, the smooth muscle guanylate cyclase. Superoxide ($O_2^{\bullet -}$) is produced by a number of sources in the vasculature, including xanthine oxidase, NADPH and NADH oxidases [3]. Intra-cellular sources of $O_2^{\bullet -}$ may include mitochondrial enzymes as well as the nitric oxide synthase systems [3]. Extracellular superoxide dismutase (SOD) consumes $O_2^{\bullet -}$ and maintains the low levels of $O_2^{\bullet -}$ seen under normal physiological conditions [3].

Both $NO^{\bullet}$ and $O_2^{\bullet -}$ are stable and relatively unreactive. However, the reaction between $NO^{\bullet}$ with $O_2^{\bullet -}$ is widely believed to occur under a number of physiological and non-physiological conditions. The product of the $NO^{\bullet}$ reaction with $O_2^{\bullet -}$ is peroxynitrite ($ONOO^-$), the conjugate base of peroxynitrious acid (HOONO) (for review see [4]).

$$NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^- \tag{1}$$

The half life of $ONOO^-$ under physiological conditions is less than 1 s, as it decomposes spontaneously to give rise to a number of oxidation products [4]. Unlike $NO^{\bullet}$, $ONOO^-$ has been shown to be a very powerful cytotoxic agent in vivo, as it can disrupt and/or destroy critical cellular processes [4]. Nitration of tyrosine residues on proteins, by $ONOO^-$ has been detected in vivo [4].

Under normal conditions, the interplay between $NO^{\bullet}$, $O_2^{\bullet -}$ and $ONOO^-$ in the vasculature is a delicate balance that must be maintained between the pro and antioxidant processes [5] (Fig. 1(a)). Moreover, $O_2^{\bullet -}$ and $ONOO^-$ have recently been shown to contribute to a state of dynamic equilibrium between oxidative and anti-oxidative mechanisms, such as endogenous glutathione, in modulating the adaptive responses in vascular system to oxidative stress [6]. An excess production of $O_2^{\bullet -}$ and/or the direct scavenging of $NO^{\bullet}$ may deplete normal levels of $NO^{\bullet}$ resulting in the loss of the beneficial effects of $NO^{\bullet}$ and the concomitant formation of $ONOO^-$.

Endothelial cells can, under a variety of physiological conditions, produce another oxidant, hydrogen peroxide, by a dismutation reaction of $O_2^{\bullet -}$, which in large part due to leakage of $O_2^{\bullet -}$ from normal electron transport systems [7].

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \tag{2}$$

Increased peroxide production is thought to occur under conditions of tissue reperfusion with oxygenated media after ischemia [7]. The basal and stimulated endothelial cell production and release of both $O_2^{\bullet -}$ and H_2O_2 are well documented [8]. Accumulation of H_2O_2 in endothelial cell culture has recently been shown to occur after reperfusion with oxygenated solutions, and the levels of H_2O_2 correlated well with the duration of ischemia [8]. Activated neutrophils and platelets are also known to contribute to the local concentration of hydrogen peroxide under the condition of ischemia and reperfusion [7].

Interaction of cell-free hemoglobin with vascular oxidants

Nitric oxide/peroxynitrite

Hypertension is one of the most commonly seen side effects upon the infusion of chemically or genetically modified hemoglobins in human subjects. This was attributed, at least in part, to the interaction of hemoglobin with endothelial derived nitric oxide [9]. Cell-free hemoglobin is a relatively small protein that can reach $NO^{\bullet}$ production sites by crossing the endothelial lining of the vessel wall, removing $NO^{\bullet}$, and inhibiting its vasorelaxing action (Fig. 1(b)). The clinical implications of hemoglobin's reaction with nitric oxide have assumed a major significance in recent years, with possible serious consequences for the development of a safe blood substitute [9]. Several alternative mechanisms to the scavenging of the vasodilator nitric oxide have also been proposed to explain the pressor effects of hemoglobin solutions. These include a possible interference of hemoglobin with endothelin, an in vivo vasoconstrictor [10], or with autoregulatory-based mechanisms which control the flow of blood and oxygen supply to tissues [11]. Although a full understanding of the etiology of the pressor effect of hemoglobin is still elusive, a role for nitric oxide/hemoglobin interaction has been confirmed by several in vitro and in vivo studies [9]. Using a specific NOS synthase inhibitor (L-NAME) prior to hemoglobin infusion had no effect on hemodynamic responses in an animal model, whereas the infusion of L-Arginine, precursor of $NO^{\bullet}$, or nitroglycerin, an $NO^{\bullet}$ donor, reduced the vasoreactivity of hemoglobin [12].

Some of the emerging experimental approaches that have been designed to resolve these unfavorable oxidative reactions of hemoglobin with $NO^{\bullet}$ include *genetic engineering*, *chemical polymerization* and *liposome encapsulation*. These result in proteins that are less reactive with $NO^{\bullet}$, or are large enough to remain longer in circulation. Mutating Leucine (B10), a key distal pocket amino acid to a larger aromatic amino acid, phenylalanine, markedly inhibit NO-oxidation of sperm whale myoglobin, a blood substitute prototype [13]. The inhibitory effects of Leu (B10) $\rightarrow$ Phe mutation in myoglobin is exaggerated in the α subunits of hemoglobin but is not seen at all in the β subunits which reflects some of the limitations of using myoglobin as a prototype for rational mutagenesis of hemoglobin [13]. Increasing the average molecular weight of tetrameric hemoglobin (from 64 kDa to an average of 320–645 kDa) in a rat model of partial exchange transfusion did not eliminate or reduce the vasoreactivity of cross-linked hemoglobin [14]. Thus, the suggestion that producing hemoglobin with a larger molecular size might retard extravasation and therefore, reduce hemoglobin's vasoreactivity, does not seem to be an entirely satisfactory solution for the pharmacological effect of cell-free hemoglobin [14]. Liposome encapsulated stroma free hemoglobin (SFH) and encapsulated cross-linked hemoglobin were recently shown to attenuate hemoglobin vasoconstriction in rabbit arterial segments [15]. Kinetic binding studies of $NO^{\bullet}$ to acellular and encapsulated hemoglobins were reported to be the same, which suggests that the lipid bilayer does not present a significant barrier to $NO^{\bullet}$. It also suggests that acellular

hemoglobin was able to gain access to both the luminal and subendothelial sites of the endothelial surface, whereas encapsulated hemoglobin, because of its large size, may encounter $NO^{\bullet}$ only at the luminal site [15].

The pro or anti-oxidant character of $NO^{\bullet}$ is dependent on the chemistry, it undergoes in a biological system, and under certain circumstances, the balance between $NO^{\bullet}/O_2^{\bullet -}$ may be channeled by way of $ONOO^-$ [16]. $NO^{\bullet}$ reacts rapidly with $O_2^{\bullet -}$ ($k = 6 \times 10^9\ M^{-1}\ s^{-1}$), to form peroxynitrite ($ONOO^-$), a reaction that has been shown to play a critical role in a variety of physiological and pathological conditions, including ischemia and reperfusion injury [4]. Indeed, recent experiments have demonstrated that L-arginine depletion of cells can trigger the switching of NOS from the production of $NO^{\bullet}$ and $O_2^{\bullet -}$ to $ONOO^-$ production [17]. More recent EPR and chemiluminesence measurements of $NO^{\bullet}$ and $ONOO^-$ generation in a post-ischemic heart by the same group of researchers, have shown that $NO^{\bullet}$ formation is increased during the early period of reflow and reacts with $O_2^{\bullet -}$ to form $ONOO^-$, which results in amino acid nitration and cellular injury [18]. We have recently studied the reactions of peroxynitrite with native and some chemically modified hemoglobins, developed as oxygen carrying reperfusion agents. The hemoglobins, irrespective of the nature of their chemical modifications underwent very rapid oxidation to methemoglobin in the presence of $ONOO^-$ [19]. This is interesting, since chemical modifications have been shown to influence the heme pocket stereochemistry and consequently, its reactions with oxidants such as superoxide, peroxide and nitric oxide [20]. The reaction of hemoglobin with $ONOO^-$ appears to occur via a direct one electron reaction pathway with methemoglobin is the only product that can be identified in the first 2–3 s of the reaction. Oxidative reactions of hemoglobin by $ONOO^-$ in the form of tyrosine nitration can occur also very early (3–6 s), primarily at the β subunits, which may contribute to the destabilization of the hemoglobin molecule [19].

Of the few published reports on the physiologic effects of infusion with some of the current generation blood substitutes, the hemoglobin-nitric oxide interaction seem to have been implicated as the basis for the frequent occurrence of gastro-intestinal symptoms in human volunteers [21,22]. One of the most frequently reported side effects is difficulty in swallowing due to interference of hemoglobin with the esophageal sphincter of Oddi function [21]. Elevation in the human pancreatic enzymes, amylase and lipase has been reported with the use of some hemoglobin preparations, and is reported to be due to $NO^{\bullet}$ inhibition by hemoglobin [22].

Removal of $NO^{\bullet}$ by cell-free hemoglobin will undoubtedly reduce a patient's own natural antioxidant mechanisms, and in effect increase the chances of $ONOO^-$ production. Once $ONOO^-$ is formed it can target both hemoglobin and the surrounding tissue. With the emergence of several potential sites for $ONOO^-$ mediated cytotoxicity, many of which were previously thought to be the sites for $NO^{\bullet}$-mediated cytotoxicity it is tempting to attribute some of the reported side effects in humans to peroxynitrite-mediated cellular injury [21,22].

Superoxide/peroxide

The production of $O_2^{\bullet -}$ from hemoglobin-bound oxygen is well recognized. This process of "auto-oxidation", is known to be accelerated under hypoxic conditions [23]. At lower oxygen tension, hydrogen peroxide, which is produced from the dismutation of $O_2^{\bullet -}$, plays a role in the oxidative reaction of hemoglobin to its ferric (met) form and ultimately its oxidative modification [23].

$$HbFe^{2+}(O_2) \leftrightarrow (HbFe^{2+} + O_2 \tag{3}$$

$$HbFe^{2+} + O_2 \rightarrow HbFe^{3+}O_2^{\bullet -} \tag{4}$$

Auto-oxidation of hemoglobin is an important concern in the use of chemically or genetically modified hemoglobins as oxygen-carrying blood substitutes. Uncontrolled and spontaneous oxidation not only limits the oxygen-carrying capacity of hemoglobin, but it can initiate a series of events that evoke toxic side effects and heme loss in addition to a build up of other reactive oxygen species [23]. Hemoglobin is maintained in its ferrous, functional state (Fe^{2+}) within the normal environment of the red cell by an efficient enzymatic machinery. The absence of this reduction system in plasma results in rapid oxidation of cell-free hemoglobin solutions in vivo [24]. In a recent study, it was shown in an ovine model of exchange transfusion that approximately 40% of the infused modified hemoglobin oxidizes to its ferric (met) form in the first 24 h after infusion [25].

Extravasation of cell-free hemoglobin into the endothelial lining of the vasculature and its proximity to these cells is thought to promote endothelial oxidative stress [26]. Indeed, the cytotoxicity of hemoglobin was found to be dependent on the rate of hemoglobin auto-oxidation and correlated with time of incubation with endothelial cells and the presence of iron chelators in the culture medium [27]. In another report, exposure of endothelial cells to ferric, rather than the ferrous form of hemoglobin was found to induce sufficient oxidative stress, to trigger the formation of oxidative stress proteins and, if stress is severe enough, cell death [28]. Hemorrhagic lesions were seen upon the infusion of polymerized hemoglobin in a stressed rat model. The lesions have the character of "small vessel vasculitis", suggesting that endothelial damage played a central role in the development of these lesions [29]. Although, the primary toxic factor was reported to be the cross-linking procedure with glutaraldehyde, hemoglobin-mediated oxidative stress cannot be ruled out [30].

These studies have clearly identified several potential candidates for the agent(s) responsible for endothelial cell toxicity, but have not directly established the critical factors. The oxidative reaction of hemoglobin with hydrogen peroxide, produced largely from endothelial cells, and/or by the auto-oxidation of its heme iron, are believed to play an important role in hemoglobin-mediated tissue damage [20]. Hydrogen peroxide can induce rapid oxidation of oxyhemoglobin ($HbFe^{2+}O_2$) to methemoglobin ($HbFe^{3+}$). Mechanistic analysis of the reaction of hemoglobin and myoglobin have revealed the formation of a higher oxidation state, the ferryl heme iron ($HbFe^{4+}$) from hemoglobin, that can be detected by optical spectroscopy, and a

transient globin-associated free radical can only be detected by EPR spectroscopy [20,31].

$$HbFe^{2+} + H_2O_2 \rightarrow HbFe^{4+} + 2OH^- \quad (5)$$

$$HbFe^{4+} + HbFe^{2+} \rightarrow 2HbFe^{3+} \quad (6)$$

$$2HbFe^{2+} + H_2O_2 \rightarrow 2HbFe^{3+}2OH^- \quad (7)$$

Hydrogen peroxide produced from the dismutation of $O_2^{\cdot -}$ or from cellular sources is consumed by hemoglobin as it cycles between ferric and the ferryl hemes in a peroxidase-like manner [32]. In spite of its transient nature, ferryl hemoglobin can peroxidize lipids, degrade carbohydrates and cross-link proteins. Ferryl hemoglobin or myoglobin have been implicated in the oxidative reactions seen upon reperfusion of ischemic tissues with oxygenated media [20,26]. The globin-based radical of ferryl hemoglobin was recently detected by EPR in normal human blood. Interestingly, the source of peroxide in blood required for the reaction was reported to be from the dismutation of $O_2^{\cdot -}$ produced via the auto-oxidation of intraerythrocytic hemoglobin, in spite of the presence of the normal blood reducing mechanisms [33]. One concern, relevant to the use of hemoglobin as a blood substitute, is that in vivo production of ferryl hemoglobin may occur under conditions of ischemia and reperfusion in patients with a diminished ability to control oxidative reactions of hemoglobin. If ferryl hemoglobin persists in tissues, it can indeed cause considerable damage. In endothelial cell culture, chemically modified hemoglobin was less effective in removing hydrogen peroxide added to the medium, than unmodified hemoglobin. This suppressed peroxidative activity of hemoglobin, due possibly to the chemical modifications, correlated with the formation of a long-lived ferryl hemoglobin which induced apoptotic cell death [34]. Using a model of endothelial cells grown on microcarrier beads, it is possible to mimic many of the biological responses of ischemia and reperfusion [8]. In this model which offers the opportunity to continuously monitor hemoglobin-mediated oxidative reactions, we were able to detect the ferryl species in a time frame that corresponded closely with peroxide production and lipid peroxidation during ischemia/reperfusion (unpublished data). Although no in vivo evidence exists to directly attribute the cytotoxicity seen with cell-free hemoglobins to the ferryl species in cell culture, several protective strategies are under consideration to control and/or reduce the levels of ferryl hemoglobin. Site directed mutagenesis has recently been used to suppress the auto-oxidation and oxidative reactions of myoglobin, a blood substitute prototype. The mutation *B*10(Leu → Phe) at the entry point to the heme has a profound effect on both the rate of auto-oxidation and oxygen affinity of myoglobin (the rate of auto-oxidation decreases 10-fold, and oxygen affinity increases 15-fold) [35]. If distal histidine, which stabilizes bound oxygen to heme, is replaced with a polar residue, this results in a substantial increase in the rate of auto-oxidation. Studies are now under way in our laboratory to evaluate whether a single or combinational mutation can be used to restrict the reactivity of myoglobins with hydrogen peroxide. Other, more direct chemical strategies are also emerging aimed at

cycling ferryl back to ferric hemes by simulating a catalase-like activity of hemoproteins using an active redox compound such as nitroxide [36], or the addition of Trolox, a vitamin E analogue known for its anti-ferryl activity [37]. Other more recent approaches include the cross-linking of the red cell enzymes, superoxide dismutase and catalase, to the hemoglobin molecule. Poly Hb-SOD-catalase was found to retard methemoglobin formation in vitro and in a rat model of intestinal ischemia-reperfusion [38].

Conclusions

Hemoglobin-based blood substitutes have been under development for over 50 years. Current hemoglobin-based products have, to a certain extent, overcome some of the traditional problems associated with older generation blood substitutes, i.e. purity, stabilization of the cell-free protein, unloading of oxygen and enhancement of its functions. However, new and more challenging problems, such as hemoglobin's effects on the vascular tone through the removal of nitric oxide and the ensuing iron-mediated free radical reactions, provide plausible mechanisms for the some of the observed toxicities associated with the current generation of hemoglobin products. The potential for oxidative damage by hemoglobin solutions opens up a critical field of study that will have to be well understood before any of the present generations of products will be successful as reperfusion agents.

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 17

in vivo Oxygenation of Deoxy-Hemolink™ Following Exchange Transfusions of 50% or 90% the Blood Volume in Rats

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Introduction

Hemoglobin based oxygen carriers have shown their potential as red cell substitutes in several clinical settings [1–8]. Stability in storage is one of the concerns in the clinical use of hemoglobin based oxygen carriers. Hemolink™ is currently stored at −80°C. This storage and rapid thawing before use in emergency conditions is problematic. Hemolink™ in its reduced form (deoxy-Hemolink™) is more stable in storage at ambient temperatures, since the conversion of deoxy-Hemolink™ to methemoglobin is greatly retarded, compared to Hemolink™ (unpublished data). However, its ability to be oxygenated in vivo needs to be demonstrated to qualify it as an oxygen carrier. The objective of the current investigation is to demonstrate in vivo oxygenation of deoxy-Hemolink™ and its physiological equivalence to Hemolink™, in terms of oxygen transport and its ability to support life in the virtual absence of endogenous red blood cells.

Materials and methods

Animals

Male Sprague Dawley rats, 300–400 g (Charles River, St. Constant, Quebec, Canada) were used for this study. The rats were acclimatized for one week after arrival to the laboratory and allowed free access to food and tap water.

Test and control solutions

Hemolink™ is an oxidized raffinose crosslinked and polymerized human hemoglobin solution [9] formulated in lactated Ringer's solution and stored at −80°C. Deoxy-Hemolink™ was prepared from Hemolink™ by charging nitrogen into the solution using a hollow-fiber oxygenator (Capiox 350, Terumo Corporation, Tokyo, Japan); it was stored in glass bottles under nitrogen at 4°C. Rat whole blood from donor rats was collected and anti-coagulated with heparin prior to the start of infusion and kept

at room temperature for up to 30 min. Hemoglobin solutions were warmed to room temperature (23 ± 2°C) and sterile-filtered through a 0.22 μm filter prior to infusion. All infusions (including rat donor whole blood) were administered at room temperature.

Treatment groups

Five treatment groups were selected for the two exchange transfusion (ET) studies. For 50% ET model (see below), three treatment groups were selected: (a) Hemolink™ (HLK, $n = 8$); (b) deoxy-Hemolink™ (deoxy-HLK, $n = 8$); and (c) rat whole blood (WB, $n = 5$). For 90% ET model (see below), two treatment groups were selected: (d) Hemolink™ (HLK, $n = 5$) and (e) deoxy-Hemolink™ (deoxy-HLK, $n = 5$).

Surgical procedure

On the day of the experiment, rats were anaesthetized with Ketaset (ketamine hydrochloride, 60 mg/kg, i.m.) and Atravet (acepromazine maleate, 2.0 mg/kg, i.m.). Both femoral arteries and the right femoral vein were cannulated using a 2.5–3.5 cm PE10 tubing connected to a PE50 tubing filled with heparin-saline solution (50 USP units heparin/ml). Two to 3.5 cm of PE10 tubing were inserted into the lower abdominal aorta via the femoral artery and into the vena cava via the femoral vein. At the end of the surgery, the incision was closed with continuous sutures. The cannulas were filled with heparin-saline solution (500 USP unit/ml) and tunneled subcutaneously to the dorsum of the neck and exteriorized. The animals were then outfitted with a rodent tethering harness and miniature feed-through swivels (Stoelting Co, IL., USA) and placed in individual metabolic cages. The surgical procedure took about 30 min. The animals were then allowed to recover for 1.5 h before treatment.

Exchange transfusion procedure

Fifty percent exchange. After the end of 1.5 h recovery period, conscious animals were subjected to the exchange transfusion procedure with one of the three test solutions. After a 30 min stabilization period, the right femoral arterial and venous cannulas were connected to a microprocessor controlled syringe infusion/withdrawal pump (Stoelting, Wood Dale, IL, USA). The test solution was infused via the venous cannula and blood was withdrawn simultaneously via the arterial cannula at a rate of 0.5 ml/min. Blood samples (0.2 ml) for blood gas and hematocrit measurements were taken from the cannulated femoral artery prior to and immediately after the completion of the exchange. Blood pressure and heart rate were continuously monitored during the exchange and for 2 h post-exchange using a pressure transducer (COBE, Lakewood, CO, USA) connected to the left femoral artery. Systolic, diastolic and mean arterial blood pressure and heart rate were recorded continuously using the Buxco LS20 system. The volume of exchange was 40 ml/kg and 50% ET was

confirmed by the reduction in hematocrit (Hct) to 50% of the pre-exchange value. The animals were sacrificed at the end of the 2-h post-exchange monitoring period by Somnotol over-dose.

Ninety percent exchange. After the end of 1.5 h recovery period, the animals were subjected to exchange transfusion with one of the two test Hemolink^{TM} solutions. The right arterial and venous cannulas were connected to a microprocessor controlled syringe infusion/withdrawal pump. The test solution was infused via the venous cannula and blood was withdrawn simultaneously via the arterial cannula at a rate of 1.0 ml/min. Blood samples (0.5 ml) for blood gas parameters, hematocrit and CO-Oximetry determinations were taken from the left cannulated femoral artery prior to exchange, immediately post-exchange and certain time points post-exchange. The volume of exchange was 120 ml/kg and 90% ET was confirmed by a Hct < 5% at the end of the exchange. The animals were kept in their metabolic cages for seven days to monitor survival. At the end of the 7-d observation period, the animals were sacrificed by Somnotol over-dose.

Observation and laboratory investigations

Hematocrit (Hct). Blood samples were collected into the heparinized capillary tubes for Hct measurements.

For the 50% exchange model, Hct of the animal was measured prior to ET and immediately post-exchange. For the 90% exchange model, only the Hct for the immediate post-exchange time point was measured.

Blood Gases. A pH/blood gas analyzer (Model AVL995-Hb Automatic Blood Gas System, AVL Scientific Company) was used for the measurements. Blood gas variables were monitored prior to exchange and immediately post-exchange for the 50% ET model and extended to 0.5, 1, 2 and 3 h post-exchange for the 90% ET model.

Hemodynamics. Blood pressure and heart rate were monitored continuously for at least 30 min prior to exchange, during exchange and for at least 2 h post-exchange for the 50% ET model. No hemodynamic measurements were made in the groups subjected to the 90% ET.

CO-Oximetry measurements. The CO-Oximetry parameters were not monitored for the 50% ET. For the 90% ET, the CO-Oximetry parameters were monitored from the stock solutions prior to exchange and from arterial blood immediately after the exchange and 0.5, 1, 2 and 3 h post-exchange using a CO-Oximeter (Model IL-682, Coulter Electronics of Canada, Ltd, Burlington, Ontario). The arterial catheter was connected directly to the tubing of the CO-Oximeter to avoid exposure of the sample to the air.

Data analysis

Results are expressed as mean±standard error of mean (SEM). Within group comparisons were performed with repeated one-way analysis of variance (ANOVA) followed by post-hoc tests using the Dunnett method. Pre-infusion values were used as the control values for statistical analysis. Differences between groups were compared with ordinary one-way ANOVA followed by post-hoc tests using Turkey method. $p < 0.05$ was taken to be statistically significant.

Results

Fifty percent exchange

Hematocrit (Hct). The hematocrit (Hct) for Hemolink™ (HLK) and deoxy-Hemolink™ (deoxy-HLK) animals are summarized in Table 1. Hct was $39.3 \pm 0.6\%$ for the HLK group prior to exchange and $20 \pm 0.2\%$ post-exchange. For the deoxy-HLK group, Hct was $38.1 \pm 0.5\%$ prior to exchange and $19.1 \pm 0.2\%$ post-exchange indicating that approximately 50% of the animal's red blood cell was removed in both groups.

Blood gases. The blood gas measurements for Hemolink™ and deoxy-Hemolink™ groups are summarized in Table 1 and depicted in Fig. 1. For both groups, there were no significant differences in pO_2, pCO_2 and pH prior to exchange and post-exchange.

Hemodynamics. The changes in mean arterial blood pressure (MAP) for the three test groups are presented in Fig. 2. Prior to exchange, MAP values were 116 ± 1, 115 ± 1 and 115 ± 1 mmHg for the whole blood control (WB), HLK and deoxy-HLK groups, respectively. MAP increased to 119 ± 1 mmHg at 15 min after the start of exchange for the WB group and dropped back to 116 mmHg at the end of exchange and remained at 113–118 mmHg throughout the entire 2 h post-exchange period. For the HLK group, MAP increased to 124 ± 1 mmHg at 15 min after the start of exchange and remained at 121–124 mmHg during exchange and throughout the entire 2 h post-exchange period. For the deoxy-HLK group, MAP increased to 121 ± 2 mmHg at 15 min post-exchange and remained at 119–121 mmHg during exchange and throughout the entire 2 h post-exchange period. The changes in MAP relative to its own baseline in the WB group were not significant, but were significant for both HLK and deoxy-HLK groups ($p < 0.01$). Comparison of inter-group difference in MAP showed that HLK and deoxy-HLK groups were not significantly different from each other at any time point.

The changes in heart rate (HR) for the three test groups are shown in Fig. 3. HR were 425 ± 5, 458 ± 11 and 437 ± 14 bpm for the WB, HLK and deoxy-HLK groups, respectively, prior to exchange. For the WB group, HR was unchanged after the start and at the end of exchange (~30–60 min), and the minor increments (20–35 bpm) were not of statistically significant magnitude. The increase in HR at any time for the

Table 1

Hematocrit and Blood Gas Data in Rats with 50% ET (Mean ± SEM, $n = 7$)

			Blood Gas Analyzer Data					
	Hct %		pH		pO_2 (mmHg)		pCO_2 (mmHg)	
Time	HLK	deoxy-HLK	HLK	deoxy-HLK	HLK	deoxy-HLK	HLK	deoxy-HLK
Prior to ET	39.3 ± 0.6	38.1 ± 0.5	7.39 ± 0.01	7.42 ± 0.01	113.1 ± 4.9	108.4 ± 3.6	44.2 ± 1.1	42.6 ± 0.5
Post ET	20 ± 0.2	19.1 ± 0.2	7.39 ± 0.01	7.43 ± 0.01	113.1 ± 4.6	100.9 ± 2.9	44.3 ± 0.7	42.2 ± 1.3

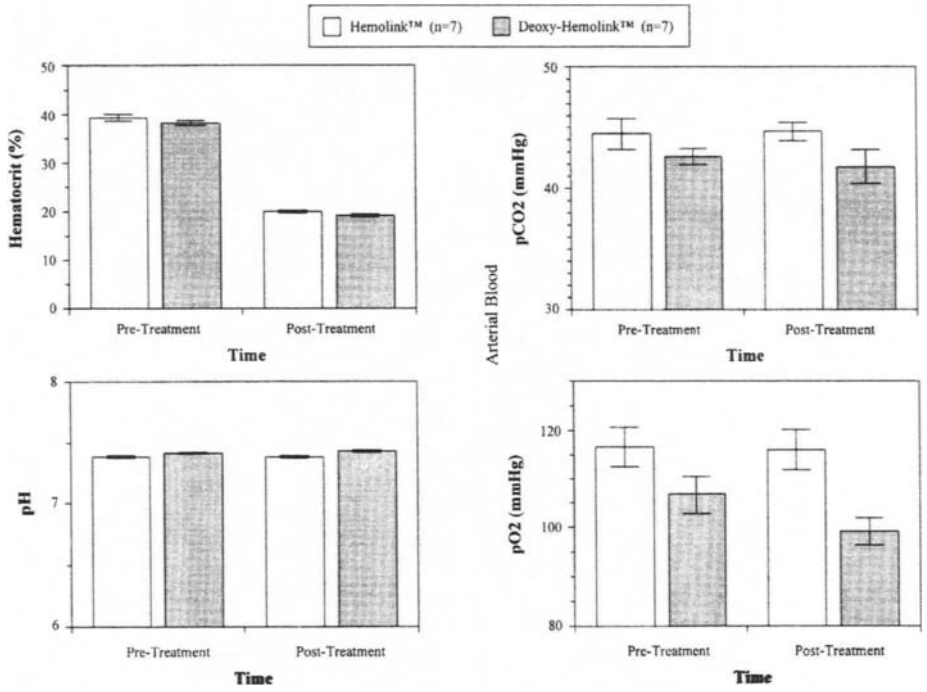

Fig. 1. Changes in hematocrit and blood gases following 50% exchange transfusion with Hemolink™ or deoxy-Hemolink™ in rats. Rats were exchanged 50% of their blood volume with either Hemolink™ (blank, $n = 7$) or deoxy-Hemolink™ (shaded, $n = 7$). Blood samples were collected prior to and immediate post-exchange for measurements of hematocrit (upper left) and blood gases including pH (lower left), pCO_2 (upper right) and pO_2 (lower right). Data are expressed as Mean ± SEM.

WB group was not significant from its pre-exchange baseline value. For the HLK group, HR decreased an average of 55 bpm at 15 min after the start of exchange and remained about 35–60 bpm lower than the baseline for the rest of the exchange and the 2 h post-exchange period. The decrease in HR from the pre-exchange baseline value for the HLK group was significant for all time points ($p < 0.05$). For the deoxy-HLK, HR decreased about 15 bpm at 15 min after the start of exchange and further decreased to about 30–35 bpm lower than the baseline at 45 min (approximately 15 min post-exchange) and remained decreased until the end of monitoring period. The difference in HR for the deoxy-HLK group prior to exchange and post-exchange was significant only at 105 min after the start of exchange ($p < 0.05$). Comparison of inter-group difference in HR showed that HLK and deoxy-HLK groups were not significantly different at any time point except at 30 min after the start of exchange when the HLK group had a more pronounced bradycardic effect than the deoxy-HLK group ($p < 0.05$).

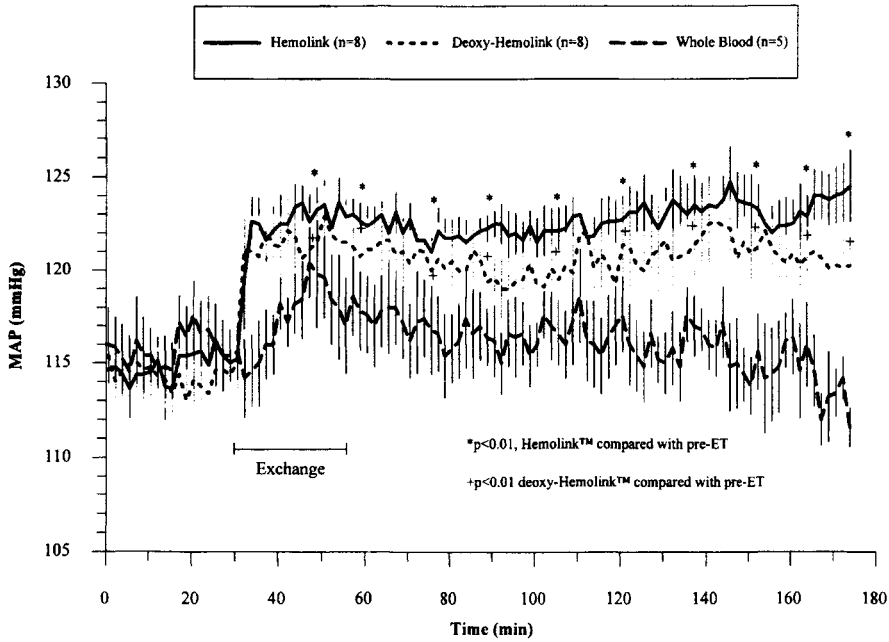

Fig. 2. Changes in mean arterial blood pressure (MAP) following 50% exchange transfusion in rats. Rats were exchanged with Hemolink™ (solid black line, $n = 8$), deoxy-Hemolink™ (dotted line, $n = 8$) or homologous rat whole blood (broken black line, $n = 5$). MAP was monitored 30 min prior to, during and 120 min post-exchange.

Ninety percent exchange

Hematocrit (Hct). Hematocrit values for the HLK and deoxy-HLK groups were all $\leq 5\%$ immediately after the exchange.

CO-Oximetry measurements. The CO-Oximeter parameters for HLK and deoxy-HLK groups are summarized in Table 2(A) and 2(B), respectively. Comparison between the two groups is depicted in Fig. 4. It can be seen that immediately after exchange, the reduced Hb (RHb) dropped from $\sim 82\%$ in the stock solution prior to ET to $\sim 25\%$ in arterial blood after 90% ET in the deoxy-Hemolink™ exchanged group. Concurrent with this decrease was the rise of the oxy-Hb (O_2Hb) level from $\sim 5\%$ to $\sim 65\%$ which remained elevated and virtually unchanged throughout the 3 h post-exchange period. This demonstrates that deoxy-HLK is oxygenated in vivo. For the HLK exchanged group, immediately after exchange, RHb increased slightly from $\sim 10\%$ (stock solution) to $\sim 20\%$ (arterial blood) at the expense of the oxy-Hb. In both cases, after exchange transfusion under physiological O_2 partial pressure, the oxy-Hb level in the circulation remained at about 70% and a 20–25% of Hemolink™ fraction remained unsaturated with oxygen. The MetHb levels for both study groups remained quite stable and were less than 10% throughout the monitoring period.

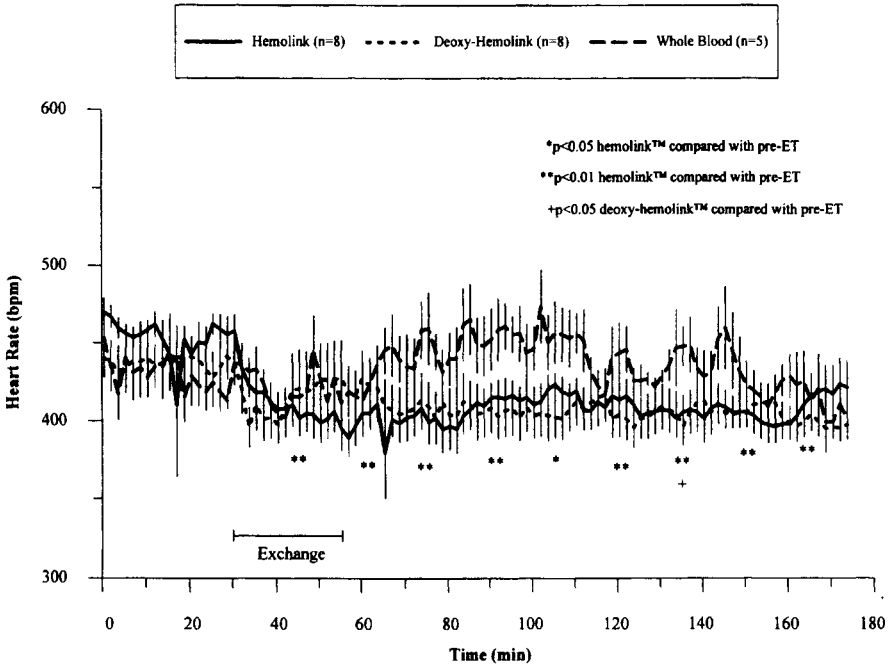

Fig. 3. Changes in heart rate following 50% exchange transfusion in rats. Rats were exchanged with Hemolink™ (solid black line, $n = 8$)), deoxy-Hemolink™ (dotted line, $n = 8$) or homologous rat whole blood (broken black line, $n = 5$). Heart rate was monitored 30 min prior to, during and 120 min post-exchange.

Blood gases. The blood gas data for HLK and deoxy-HLK groups are summarized in Table 2(A) and 2(B), respectively, and graphically compared in Fig. 5. There were no significant differences in pH, pO_2, and pCO_2 between the two groups prior to exchange and post-exchange suggesting that deoxy-HLK behaves similarly to HLK following 90% exchange. The bicarbonate was also not different between the two groups (Fig. 5).

Survival. After 90% exchange transfusion with either HLK or deoxy-HLK, all animals survived the 7-d observation period.

Discussion

Unpublished data demonstrate that Hemolink™ in its deoxygenated form is more stable in storage. The increase of methemoglobin in deoxy-Hemolink™ with time in storage at 4°C or room temperature is greatly retarded compared to that in Hemolink™. This enhanced temperature stability of deoxy-Hemolink™ makes it more feasible for its use in clinical and emergency situations.

Table 2

CO-Oximeter & Blood Gas Data in Rats with 90% ET (Mean ± SEM, $n = 5$)

(A) Hemolink™ Group

	CO-Oximeter Reading						Blood Gas Analyzer Data		
Time	THb (%)	$\%O_2Hb$	%COHb	%MetHb	%RHb	O_2CT (vol%)	pH	pCO_2	pO_2
Prior to ET	10.1 ± 0.20	79.3 ± 1.61	0.1 ± 1.14	11.1 ± 0.63	9.1 ± 0.32	11.1 ± 0.41	7.39 ± 0.01	45.5 ± 1.41	108.2 ± 3.67
0 hr Post ET	10.1 ± 0.12	68.9 ± 0.79	2.7 ± 0.85	8.9 ± 0.36	19.5 ± 0.36	9.7 ± 0.22	7.38 ± 0.01	46.3 ± 0.82	113.1 ± 4.86
0.5 hr Post ET	9.9 ± 0.25	70.5 ± 0.81	0.7 ± 0.55	8.7 ± 0.30	20.1 ± 0.40	9.7 ± 0.34	7.44 ± 0.01	44.7 ± 1.11	112.6 ± 1.15
1 hr Post ET	9.5 ± 0.10	70.7 ± 0.10	0.1 ± 0.10	8.6 ± 0.10	20.7 ± 0.60	9.3 ± 0.10	7.44 ± 0.10	45.6 ± 0.10	107.0 ± 1.34
2 hr Post ET	9.4 ± 0.22	71.0 ± 0.09	−0.6 ± 1.12	8.7 ± 0.40	20.8 ± 0.52	9.2 ± 0.19	7.46 ± 0.01	46.9 ± 1.71	109.0 ± 1.94
3 hr post ET*	9	70.5	−0.8	8.6	21.7	8.8	7.5	44.5	103.3

(B) Deoxy-Hemolink™ Group

	CO-Oximeter Reading						Blood Gas Analyzer Data		
Time	THb (%)	$\%O_2Hb$	%COHb	%MetHb	%RHb	O_2CT (vol%)	pH	pCO_2	pO_2
Prior to ET	10.4 ± 0.08	4.5 ± 0.59	7.1 ± 0.30	6.0 ± 0.57	82.3 ± 0.38	0.7 ± 0.08	7.40 ± 0.01	44.8 ± 0.88	103.3 ± 1.99
0 hr Post ET	10.5 ± 0.05	66.4 ± 1.02	2.4 ± 0.37	5.7 ± 0.92	25.6 ± 0.33	9.7 ± 0.16	7.41 ± 0.00	46.1 ± 0.87	108.2 ± 2.05
0.5 hr Post ET	10.3 ± 0.10	67.1 ± 1.01	0.6 ± 0.45	6.1 ± 0.93	26.2 ± 0.44	9.6 ± 0.16	7.45 ± 0.01	45.2 ± 0.82	103.9 ± 1.02
1 hr Post ET	10.1 ± 0.21	68.0 ± 0.88	0.4 ± 0.85	6.6 ± 0.85	25.4 ± 0.43	9.6 ± 0.18	7.46 ± 0.01	44.7 ± 0.68	107.2 ± 3.22
2 hr Post ET	9.9 ± 0.24	67.0 ± 0.78	−0.4 ± 0.21	8.0 ± 0.58	25.3 ± 0.12	9.3 ± 0.23	7.45 ± 0.01	46.8 ± 0.40	101.4 ± 2.03
3 hr post ET	9.5 ± 0.25	67.7 ± 0.52	−0.4 ± 0.18	8.5 ± 0.56	24.2 ± 0.61	9.0 ± 0.32	7.47 ± 0.00	45.9 ± 0.58	105.1 ± 0.46

Note: The CO-oximeter readings corresponding to prior to ET are the values for the stock solutions before ET.
*$n = 1$.

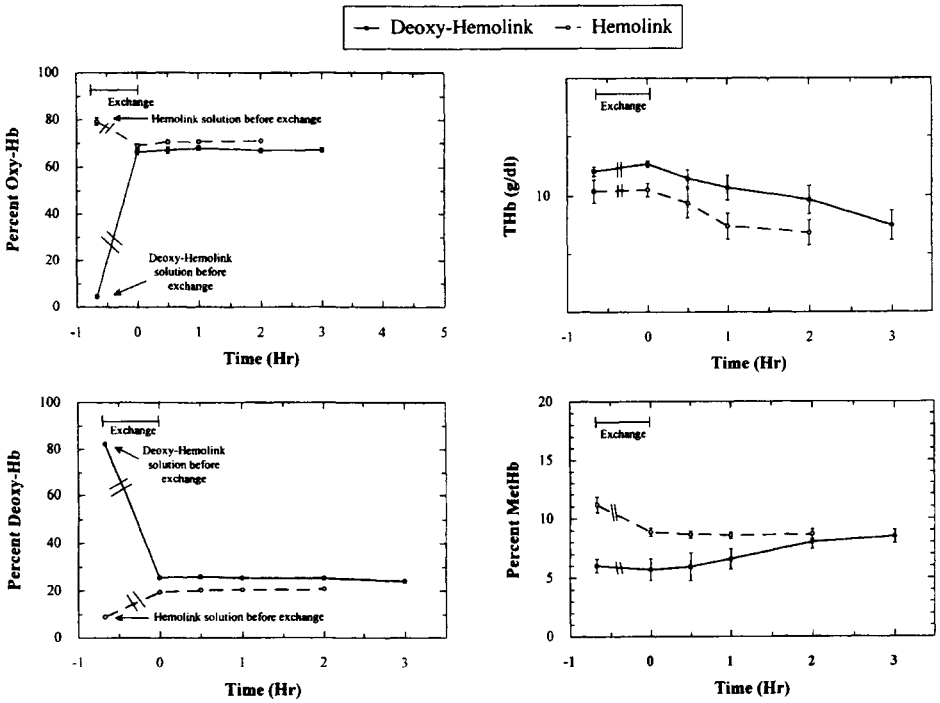

Fig. 4. Changes in CO-Oximetry parameters following 90% exchange transfusion in rats. Rats were exchanged with Hemolink™ (broken line, $n = 5$) or deoxy-Hemolink™ (solid line, $n = 5$). Blood samples were collected prior to, and 0.5, 1, 2 and 3 h (deoxy-Hemolink™ only) post-exchange. Upper left: percent of oxy-Hb, lower left: percent of deoxy-Hb, upper right: total Hb, lower right: percent of metHb.

In this study, in vivo oxygenation of deoxy-Hemolink™ was assessed in the 90% ET model. The procedure for exchange took 30–40 min to complete depending on the body weight of the animal. By the end of the exchange and through the subsequent 3-h monitoring period, there was no difference in the in vivo oxy-Hb level in arterial blood between deoxy-Hemolink™ and Hemolink™ groups. This study shows that once introduced in the circulation, deoxy-Hemolink™ and Hemolink™ are oxygenated in the lungs to a similar extent.

Comparison of hemodynamic response to Hemolink™ and deoxy-Hemolink™ was conducted when half of the animal's blood was replaced with either solution. A mild increase in mean arterial blood pressure and a bradycardia were observed in both groups with no quantitative difference between them. Scavenging of nitric oxide (NO) by heme is one of the mechanisms used to explain the pressor effect induced by hemoglobin based oxygen carriers. If this is the case, then the NO binding affinity of Hemolink™ and deoxy-Hemolink™ appears to be the same.

Apparently, adequate ventilation was maintained in both 50% and 90% ET

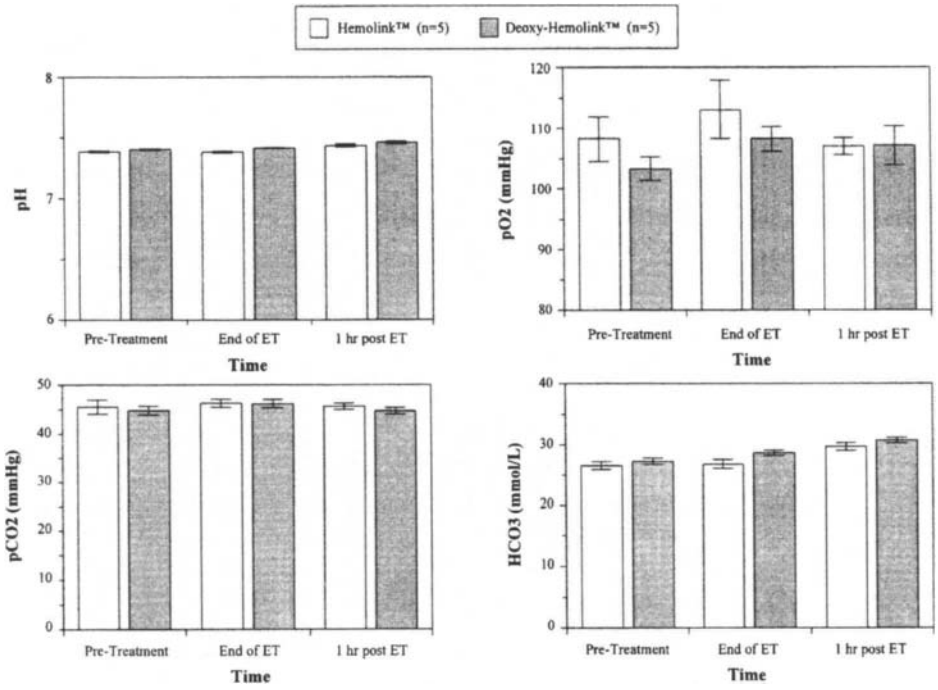

Fig. 5. Changes in blood gases following 90% exchange transfusion in rats. Rats were exchanged with Hemolink™ (blank, $n = 5$) or deoxy-Hemolink™ (shaded, $n = 5$). Blood samples were collected prior to ET, at the end of ET and 1 h post-exchange. Upper left: pH, low left: pCO_2, upper right: pO_2, lower right: HCO_3.

models, as shown by arterial pO_2 measured within the normal range after ET. The ability to support life and prolonged survival indicate efficacy in maintaining adequate oxygen supply to the tissues, during the period (approximately 2 d) when sufficient concentration of Hemolink™ is retained in the circulation and before hemopoiesis begins to restore red cell mass.

Besides, the pO_2, pCO_2, bicarbonate and pH for the Hemolink™ and deoxy-Hemolink™ animals were also indistinguishable suggesting that both products behave physiologically in a similar fashion following administration. The low methemoglobin levels in both groups throughout the 3-h monitoring period indicate that the two solutions are stable in the circulation.

We notice that at presumably normal alveolar pO_2s, Hemolink™ is not as fully saturated as whole blood would be. It is well-known that crosslinking and polymerization can change the oxygen affinity and may eliminate cooperativity of native hemoglobin [10–14]. Such seems to be the case with Hemolink™. Its $p_{50}(= 35)$ is somewhat higher than and its n value $(= 1)$ is lower than that of intraerythrocytic hemoglobin. Following in vivo administration, about 80% of the oxygen saturable

fraction of Hb (total Hb–metHb) is oxygenated in the arterial blood in both Hemolink™ and deoxy-Hemolink™ groups. About 20–25% of total Hb fraction remain unsaturated. We believe that the low oxygen affinity and the modification of heme pocket play roles in keeping this small fraction unoxygenated in circulation. However, the exact mechanism whereby this fraction apparently remains unsaturated at ambient alveolar pO_2, and its impact, are unclear at present and remain to be investigated.

Conclusions

Deoxy-Hemolink™ prepared for greater stability in storage, appears to behave similar to Hemolink™ once administered in vivo. It is oxygenated to the same level as Hemolink™ in the lungs and the resulting product appears to be physiologically similar to Hemolink™ in both 50% and 90% rat exchange transfusion models. No major differences in hemodynamic responses, blood gas and CO-Oximetry parameters and 7-d survival were observed between these two products. The bioequivalence of Hemolink™ and deoxy-Hemolink™ and their efficacy in supporting life in the virtual absence of red blood cells are demonstrated by the present investigation.

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 18

Safety and Efficacy of Hemoglobin Modified by Crosslinking or Polymerization

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Introduction

Hemoglobin in the red blood cell is responsible for oxygen transport from the lungs to the tissues. In 1933 Amberson and coworkers [1] showed by exchange transfusion in cats that hemoglobin outside the red blood cell can also transport oxygen. However, after application in humans problems with kidney function were observed [2]. In 1967 Rabiner and coworkers [3] showed that improved purification of the Hb solutions by filtration could prevent kidney problems in dogs. In 1978 Savitsky et al. [4] performed a clinical safety trial in 8 healthy human volunteers with an Hb solution prepared according to the Rabiner method. Abdominal pain was reported in 2 volunteers, bradycardia in 7 and oliguria in 8. Thus, again, kidney function appeared to be affected. This observation caused stagnation in the development of such solutions. In the eighties it became clear that free hemoglobin has several properties making it unsuitable for transfusion. Firstly, the affinity of free Hb for oxygen is different. Secondly, the vascular retention time is very short and thirdly, the maximal attainable concentration is about half that in blood. Chemical modification is therefore required to improve these properties. A proper modification can also circumvent the kidney problems mentioned above. The potential advantages of Hb solutions as blood substitutes are the following:

1. The solutions can be used immediately without the need for blood grouping;
2. The solutions can be stored in a ready-for-use form for years; and
3. The solutions can be heat-treated, thus eliminating the risk of virus transmission.

Especially the latter property was an important stimulus for intensified research after the discovery of the AIDS virus in 1983. As shown above, it was important to find a suitable modification of Hb both from the point of view of efficacy and of safety.

Modification of hemoglobin improves safety and efficacy

The hemoglobin molecule consists of four chains: two α and two β chains. Inside the erythrocyte the affinity for oxygen is regulated by its internal environment, particularly by pH and 2,3-DPG. The 2,3-DPG molecule can bind reversibly between both β chains. This binding lowers the affinity of Hb for oxygen and thus improves the ability of Hb to deliver oxygen to the tissues. Outside the erythrocyte the Hb molecule can no longer interact with the internal environment, e.g. with 2,3-DPG. This means an increase in oxygen affinity causing a delivery of oxygen at a lower oxygen tension. This can give rise to problems in case other factors that determine oxygen transport are compromised, e.g. under conditions of stagnant flow. A second change is that the free Hb tetramer can dissociate into two $\alpha\beta$ dimers which can leak through the kidneys. This elimination through the kidneys is an important cause of the short vascular retention time and also a cause for toxicity to the kidney. Bunn et al. [5] showed in 1969 that loss of Hb through the kidneys can be prevented by intramolecular cross-linking of the molecule. The compound used for this purpose, however, caused a further increase of the oxygen affinity. Modification of Hb by coupling with pyridoxalphosphate (Benesch et al., 1972) [6] improved oxygen affinity, but did not prevent leakage through the kidneys.

In the CLB division of the Sanguin Blood Supply Foundation in Amsterdam we used 2-Nor-2-formylpyridoxal 5′-phosphate (NFPLP) [7] for the intramolecular coupling of the two β-chains according to Benesch et al. (1975) [8]. This was carried out from 1980–1986 in a project supported by Medigon (The Dutch Foundation or Medical Research). By means of this modification to HbNFPLP both a decrease in oxygen affinity [9] and a prolongation of the vascular retention time [10] by a factor of 3 could be achieved. Moreover, accumulation of Hb in the tubuli of the kidneys [11] was eliminated. The decrease in oxygen affinity was observed in vitro as an increased p_{50}. In liver perfusion experiments the crosslinking resulted in improved tissue perfusion and oxygenation as shown by a higher oxygen tension and a lowered tissue redox level. In 1987 Snyder et al. [12] showed that it was also possible to cross-link the two α-chains and to obtain a comparable improvement of in vivo characteristics. The in vivo half life in man was estimated to be between 6 and 10 h. This is reasonable for most clinical applications, but is not long enough in situations in which repeated infusions are not possible.

Further prolongation of the vascular retention can be obtained by polymerization of the Hb molecules [13]. Polymerization was carried out by the use of glutaraldehyde resulting in PolyHbNFPLP. This treatment couples the Hb molecules to each other resulting in a mixture of smaller and bigger polymers. Due to the increase in molecular size the retention time in the circulation is prolonged. Polymerization meanwhile lowers the oncotic activity by decreasing the number of particles. The oncotic activity determines the maximum attainable concentration of free (unmodified) Hb in the circulation: about 7 g/100 ml, about half the value of Hb in normal blood. By polymerization this iso-oncotic concentration can be increased up to

14 g/100 ml. Polymerization has the disadvantage that it increases viscosity. An optimal compromise has to be established. In the CLB a method of intermolecular polymerization with glutaraldehyde has been developed that yields a mixture of polymers at an iso-oncotic concentration of 10 g/dl with a viscosity lower than that of blood. The vascular retention time in rabbits is 24 h; in humans the retention time is estimated to be about 48 h. More recently, conditions were found under which the treatment with NFPLP could be omitted.

With a lowered oxygen content during polymerization glutaraldehyde provides both the intra- and the intermolecular bonds within and between the Hb molecules. The polymerization conditions, when chosen properly, will also produce the required oxygen affinity. The final protocol describes a simple and inexpensive production process, consisting essentially of one modification step of the Hb molecules. This approach eliminates time consuming purification methods of intermediate products.

Glutaraldehyde-polymerized Hb: PolyHbXl

Modification by polymerization with glutaraldehyde was evaluated in order to achieve a prolonged retention time in a project financed by the Dutch Ministry of Defence. The main reason for polymerization was the longer half-life will making this product particularly suitable for use in mass casualty care. Polymerization of Hb to polyHbXl with an average molecular mass of about 300 kD was considered to be optimal: a vascular half-life of 15 h in rats, an iso-oncotic concentration of 9 g Hb/100 ml and a viscosity of 1.2 cP, equal to plasma viscosity (Table 1). In man the half-life will be more than 24 h. We concluded that polyHbXl is the most suitable modified Hb to meet the requirements of a red cell substitute to be used as a resuscative fluid under military or civil emergency conditions. During the period from 1988 until 1993, the efficacy and safety of this product was investigated in small

Table 1

The main characteristics of the used batch PolyHbXl

Hemoglobin concentration	9.5 g/100 ml
Methemoglobin content	< 10%
Molecular size distribution	
64 kD	24%
128–500 kD	48%
> 500 kD	28%
Dissociable 32 kD αβ-chains	< 2%
Oncotic activity	20 mmHg
Viscosity at 37°C	1.2 cP
Oxygen affinity (p_{50})	25–30 mmHg
Phospholipids	< 0.1 nmol/ml
Endotoxin (Limulus assay)	< 0.23 EU/ml
Pyrogenicity (rabbit pyrogen test)	pass

animals. A preclinical study in pigs and monkeys in 1994 completed these observations. This chapter will deal with the main results of these studies.

Preclinical safety studies of PolyHbXl

Hemodynamic effects in pigs

Purpose of the study. We investigated the effects in pigs during and after resuscitation from haemorrhagic shock with PolyHbXl or autologous blood. Furthermore, systemic oxygen transport and regional blood flow were measured and possible toxic side effects were evaluated.

The model. This was based on the model developed by Hess et al. [15] intended for toxicity testing of hemoglobin solutions in military situations. A controlled hemorrhagic shock was induced by withdrawal of about 35% of the circulating blood volume. After a hypovolemic period of 30 min, PolyHbXl or autologous blood was infused at the same dose of 25 ml/kg.

Before, during and after withdrawal and after infusion, the following hemodynamic parameters were determined: systemic and pulmonary blood pressure, the pressure in the left ventricle and the right atrium and the cardiac output (CO). At 4 time points, radioactively labelled microspheres were injected in order to measure the regional blood flow. Blood gases, pO_2 and pCO_2, were determined, the Hb content in the circulation, as well as pH, lactate and catecholamines in the plasma. The urine was collected and the Hb conc., Na^+ conc., and the *N*-acetyl-β-D-glucosaminidase (NAG) activity in the urine were determined.

Results

The shock period. Withdrawal of blood resulted in a decrease of MAP from 95 to 65 mmHg (Table 2), an increase in the heart rate (HR) from 125 to 200 bpm, a decreased cardiac output and blood flow through the organs with the exception of the heart, the brain and the adrenals, and an increase in the concentration of lactate and of catecholamines.

Systemic hemodynamics after infusion. After infusion of PolyHbXl or autologous blood the blood pressure rose to 105 and 100 mmHg (Table 2), respectively; in the PolyHbXl group the MAP tended to be higher, although this was only statistically significant at 150 min after resuscitation. At this time point, the systemic vascular resistance was increased (33%) above the baseline value. The HR returned to the starting values for both groups.

Systemic hemodynamics in the pig model

From the other measurements, the most striking result was the difference in arterial pressure in the pulmonary system, being higher in the PolyHbXl group than in the

Table 2

Changes of systemic hemodynamics in the pig model

Group	Control	Shock	30 min[a]	150 min[a]
MAP (mmHg)				
A	92 ± 6	60 ± 12	100 ± 12	88 ± 7
P	95 ± 7	58 ± 14	105 ± 15	105 ± 16
HR (beats/min)				
A	129 ± 17	195 ± 44	143 ± 32	142 ± 34
P	124 ± 20	204 ± 27	136 ± 25	140 ± 25
MPAP (mmHg)				
A	25.8 ± 3.6	20.7 ± 3.4	27.2 ± 4.6	26.2 ± 5.0
P	24.2 ± 3.5	21.3 ± 6.1	44.0 ± 11.0	39.5 ± 8.4

[a]After resuscitation, MAP: mean arterial pressure, HR: heart rate, MPAP: mean plumonary pressure, A: Autologous blood ($n = 6$); P:PolyHbXl group ($n = 6$). Values are means ±SD.

control group. Furthermore, an increase was observed from the starting value of 24.2 ± 3.5 mmHg to 39.5 ± 8.4 mmHg (Table 2).

Systemic oxygenation. After the infusion of PolyHbXl the arterial pO_2 decreased slightly from 124 mmHg to 114 mmHg (control group 150 mmHg). The saturation decreased from 94% to 85%; the value of the control group (autologous blood) was 93%. This was reflected in a difference in O_2 supply in the two groups. However O_2 consumption values were equal in the two groups. This is in accordance with the observation that the mixed venous pO_2 was significantly lower in the PolyHbXl group: 25 ± 6 vs 35 ± 4 mm Hg.

Blood flow through tissues and organs. The blood flow through the lungs had decreased in the PolyHbXl group 15 min after infusion as compared with the control group but no further significant differences were observed between the PolyHbXl group and the control group.

There was no difference in the decrease of the initially increased levels of lactate and the catecholamines in the two groups. Both these parameters (used as markers for shock) decreased rapidly to control values. The difference in the counts of peripheral blood cells between both groups was restricted to that expected on the basis of the hemodilution by PolyHbXl.

Diuresis and kidney function. No Hb was detectable in the urine and there was no increase in creatinine or NAG excretion. The diuresis during resuscitation was significantly lower after administration of PolyHbXl (50 ± 28 ml vs 144 ± 43 ml).

Discussion. After hemorrhagic shock and a short hypovolemic period the hemodynamics recovered well after infusion of PolyHbXl. This is also the case for the

systemic oxygen transport. The lower arterial saturation and O_2 supply in the PolyHbXl group did not result in a difference in O_2 consumption.

However, there is an increased pressure in the pulmonary system. This observation should be taken into account, particularly in patients in whom lung function problems can be expected. There were no signs of kidney toxicity since no influence on kidney function was found.

Effect on blood pressure in the anesthetized rat

Purpose. Quantification of the effect by determination of dose response curves after infusion of PolyHbXl and of gel chromatography subfractions, a monomer fraction (MMF, Mw = 64 kD (95%)) and a "polymer fraction" (PMF, Mw > 124 kD). Investigated was whether:

1. The effect was related to molecular size.
2. An interaction with NO was involved in the increase in blood pressure (BP).

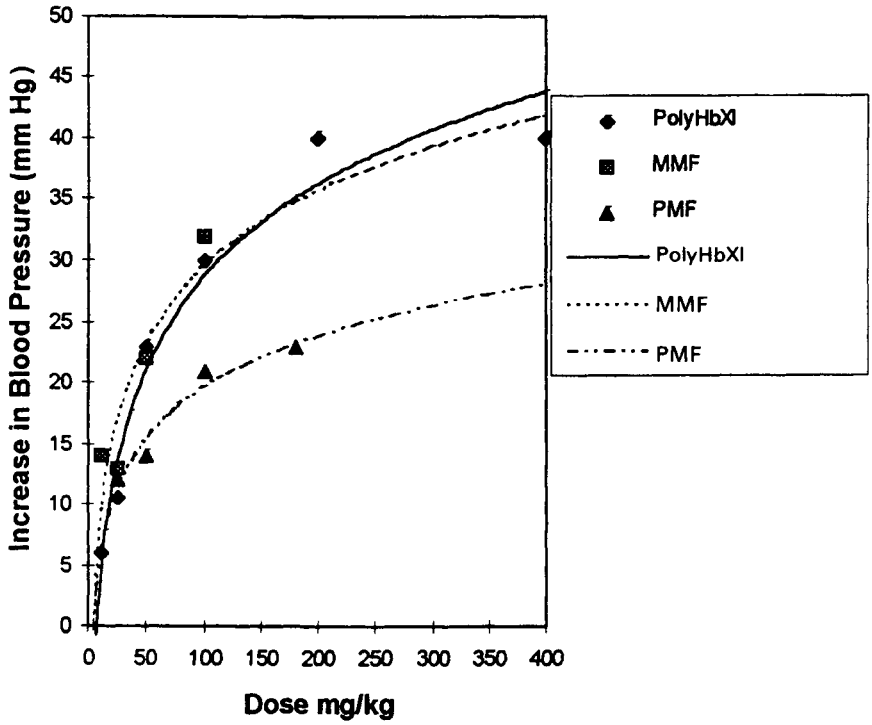

Fig. 1. Changes in blood pressure of the anesthetized rats after infusion of polyHbXI and of gel chromatography subfractions, a monomer fraction (MMF, Mw = 64 kD) and a polymer fraction (PMF, Mw > 124 kD).

Model. Rats of about 250 g, anesthetized by Hypnorm (fentanyl and fluanison) were used. A cannula was introduced in the jugular vein for infusion of the solutions and a cannula in the carotid artery for the registration of BP and heart rate. After a period of 15 min with a stable signal, 1 ml of test solution was infused at a rate of 1 ml/min.

Results. From the curves in Fig. 1 the effect at a dose of 400 mg/kg was estimated, which was considered to be the maximum effect. For PolyHbXl and MMF about 45 mmHg, for the polymer fraction PMF about 30 mmHg. The assumption that the value of 45 mm Hg (about 45–50% of the starting value) is close to the maximum is also based on data obtained after exchange transfusions in the same model, when an increase in BP of about 50% usually is observed at a dose of 3.5 g/kg. The increase in BP concurred with a decrease in heart rate from 10 to 20%. The maximum effect was observed after 5 min, the speed of the response was independent of the dose. The effect lasted maximally 1 h for a dose of 400 mg/kg. The half maximum effect was observed at a dose of about 65 mg/kg; which was nearly equal for all tested solutions. CyanometpolyHbXl, the oxidized form of Hb to which cyanide is bound and which cannot bind O_2, CO or NO, only produced an increase of BP at doses of more than 800 mg/kg. Infusion of nitroarginin, a compound, that inhibits the synthesis of NO by the endothelial cells, also caused an increase in BP. The maximum effect resembled that of PolyHbXl. After infusion of nitroarginin, a subsequent infusion of PolyHbXl had hardly any effect at a dose of 200 mg/kg. The increase in BP by PolyHbXl could be counteracted with a low dose of nitroglycerine, 50 µg/kg.

Effect on blood pressure in conscious rat

Model. Relatively low doses of Hb solutions (50-400 mg/kg) were infused to rats of about 250 g with a cannula in the carotid artery and without anesthesia.

Results. A small dose PolyHbXl of 50 mg/kg resulted in a rapid rise in BP of 15 mm Hg (about 10% of the starting value). With doses between 200 and 400 mg/kg the maximum was virtually reached. The theoretical (calculated) maximum rise was 40 mm Hg (about 30% of the starting value). The maximum rise in the conscious rats was lower than in the anesthetized rats (30% vs 50%). The duration of the effect was longer than in the anesthetized rat; i.e. between 60–120 min.

Conclusions. The data support the hypothesis that this rise in BP is mediated mainly by the binding of NO by the Hb molecules themselves, while NO functions as EDRF (endothelial derived relaxation factor). The small, albeit significant difference in effect of the polymer and monomer fractions does not support for the supposition that 64 kD Hb molecules are the only molecules that influence the BP. Anesthesia influences the extent of the BP effect.

Acute toxicity in the rabbit

Purpose and model. The purpose of this study was to detect any toxic effects during 8 d. Included was a group of animals (I) that received PolyHbXl contaminated with endotoxin, whereas group II received PolyHbXl. This combination was chosen because small amounts of endotoxin have been reported to potentiate the toxic effects of hemoglobin. Control experiments were performed with human albumin (Group III). Three groups of 6 rabbits received light anesthesia and a small amount of their blood was taken (15 ml/kg); the rabbits received 20 ml/kg PolyHbXl or albumin. After 1 h, the rabbits were placed in metabolic cages.

Results. A small decrease in body weight occurred in all groups after the animals were put into the cages and 1 d after the exchange transfusion. Subsequently, growth rates became normal.

Hematology. In group I a significant increase in the leukocyte count after 1 h with a maximum on day 2. No difference between PolyHbXl and albumin was observed.

Clinical chemistry. A slight increase in ALT and gamma-GT on day 2; also a slight increase in plasma creatinine. One animal in the PolyHbXl group (II) which died between day 4 and 5 had a decreased number of erythrocytes and thrombocytes on day 3 and a significantly increased number of leukocytes. Autopsy revealed intestinal bleeding. No pathological abnormalities were observed further. The PolyHbXl group showed a light red color on the liver without other macroscopic abnormalities. PolyHbXl caused a short-lasting rise in blood pressure of about 20%.

Discussion. The slight decline in body weight observed in all rabbits was probably due to the anesthesia. Changes in hematology are in accordance with the hemodilution. The increaese in enzymes in the rabbit plasma is small.

Conclusion. PolyHbXl does not cause serious detrimental effects when compared with the control group, human albumin, also not in the presence of small amounts of endotoxins. We could not determine whether the death of one rabbit was related to the administration of PolyHbXl.

Kidney toxicity in a hemorrhagic rat model

Purpose. Investigation of acute effects of purified and modified hemoglobin solutions on kidney function, particularly of parameters depending on integrity of glomeruli amd tubuli.

Model. Anesthetized rats with a cannula in the carotid artery, in the jugular vein and in the urinary bladder were used. The GFR (glomerular filtration rate) and RPF (renal plasma flow) were determined by measuring the clearance of inulin and para-aminohippuric acid. Furthermore, measurement of blood pressure, urine output, hemoglobin concentration in the plasma and the urine and enzyme secretion (NAG, *N*-acetyl-β-D-glucosaminidase, as a marker of damage to the tubuli) was carried out. The hypovolemic model was as follows: blood was withdrawn at 1 ml/min until the blood pressure had decreased to 60–65% below the starting value, followed by a hypovolemic period of 30 min.

Animals of group 1 received human albumin 5g/dl (HSA), $n = 5$; those of group 2, hemoglobin, SFHb, $n = 5$; those of group 3, PolyHbXl, $n = 18$. HSA and SFHb (stroma free hemoglobin) were used as control solutions, the latter because free Hb was expected to cause kidney damage.

Results. The urine flow (control value 6–8 µl/min) stopped during the hypovolemic period but rose to 163 µl/min after infusion of SFHb. It stopped again at the end of the experiment. After infusion of PolyHbXl, the urine flow increased to 120 µl/min and returned to the control value in 60 min.

The MAP (mean arterial blood pressure) was lower than 35% of the control value after the withrawal of blood and increased to 150–155% after infusion of SFHb or PolyHbXl. In both situations the MAP returned to starting values within 1 h, but continued to decrease after SFHb infusion. After HSA infusion the MAP rose up to 85% of the starting value.

The glomerular filtration rate (GFR) and renal plasma flow (RPF) decreased after resuscitation with SFHb. Resuscitation with PolyHbXl caused a slight decrease of the GFR and RPF, but after 1 h both parameters returned to control levels.

During the control period NAG was secreted to an activity level of 0.63 ± 0.11 U/ml in the urine. After infusion of SFHb the NAG activity rose to 15.1 ± 2.4 U/ml, decreased slowly, but did not return to the control value. After infusion of PolyHbXl and HSA infusion an increase to 5.1 ± 1.4 and 7.4 ± 1.6 U/ml was observed, respectively. Within 45 min the amount of secreted NAG was equal to the starting value.

With regard to leakage of Hb through the kidneys; after SFHb resuscitation 40% of the infused amount of Hb was found in the urine. After infusion of PolyHbXl less than 1% was detected in the urine.

Conclusion. A purified and unmodified Hb solution impairs renal function after haemorrhage and shock. Whether this is due to obstruction of the kidneys by the leaking $\alpha\beta$ dimers is not clear. In the same model without a period of shock no damage was observed after infusion of SFHb.

PolyHbXl caused a slight decrease in RPF, but there was no sign of damage to the kidney, even not in the hypovolemic model. The tested preparation contained 2–3% non-modified (dissociable) Hb. Thus 2–3% non-modified Hb appears to be an acceptable level of contamination of PolyHbXl.

Thrombogenicity in a guinea-pig model

Purpose. In the past it has been shown that Hb solutions can activate the coagulation system [3]. This model has been developed in order to assess the thrombogenic risks of Hb solutions.

Model. We used a guinea-pig non-stasis model developed in our laboratory [16] in which we can follow the generation of fibrinopeptide A (FPA). This is one of the most sensitive markers for the activation of thrombin in vivo. The sensitivity of the model for Hb solutions was increased by infusion of a small dose of factor Xa. PolyHbXl and SFHb were tested with HSA as a control solution.

Results. No activation was detected after infusion of PolyHbXl or HSA. Only in the SFHb group a small rise of FPA was found: 5 ng/ml (control values: lower than 2 ng/ml; maximum (lethal) values: about 100 ng/ml). This rise is probably due to the presence of a small amount of phospholipid in this solution.

Conclusion. This batch of PolyHbXl was not thrombogenic. This may be extended to a general statement: properly purified Hb solutions do not activate the coagulation system.

Acute toxicity in the monkey

Purpose. To assess acute toxicity of PolyHbXl in the first week after transfusion and to examine the antibody response against potential neo-epitopes after repeated application.

Model. Rhesus monkeys, 7–9 kg, were used. Under anesthesia 10 ml/kg blood was drawn and shortly later 20 ml/kg test solution was infused: 5 monkeys received PolyHbXl and 5 control monkeys human albumin (HSA). During week 1 the following parameters were followed: clinical condition, blood pressure, ECG and blood samples were analyzed ($t = 30$ min, 1, 2 and 5 h; 1, 2, 3 and 7 d). For the the measurement of the antibody response, every week 1 ml/kg of a test solution was applied and samples of blood were taken. All exchange transfusions and sample collections were performed under anesthesia; the first intervention lasted about 3.5 h.

Results. General. Four of the 10 monkeys had a low blood pressure at the start of the experiment: MBP < 50 mmHg and all monkeys had a hypotensive reaction after the bleeding of 10 ml/kg. Seven monkeys had a mean blood pressure of less than 50 mmHg during 10–30 min. All the monkeys of both groups had granulocytosis with a maximum at 5 h, when there was an increased level of IL-6 and a slight increase in the level of fibrinogen. Furthermore there was a reversible slight increase of ALT (alanine aminotransferase) in the plasma.

The hemodilution. In the two groups of animals the exchange transfusions caused an equal decrease of the hematocrit (HSA: from 0.39 to 0.25; PolyHbXl: from 0.41 to

0.27). The plasma hemoglobin level in the animals which received PolyHbXl was 4.4 g% (37% of the total Hb). The total Hb content in the HSA group was 8.2 g% vs 11.8 g% in the polyHbXl group. The mean half life of PolyHbXl in the circulation was 28 h (large polymers about 48 h, the 64 kD fraction about 12 h). The recovery of the red cell counts showed a similar time course; after 28 d it rose to above 90% of the control values. The reticulocyte count had increased at days 7 and 14.

Toxic effects, clinical symptoms. One monkey of the PolyHbXl group appeared to be ill during two days after the infusion and bled at the anus on day 2.

Laboratory analysis showed there was a reversible rise in the creatinine level on day 3, i.e. a value of more than 13 times the control level. It normalized on day 14. There was a concurrent decrease in Na^+ and Cl^- conc. in the plasma (less than 140 mmol/l and 100 mmol/l respectively on day 2 and 3). There was reversible thrombocytopenia, starting after 5 h; the platelet count decreased from 347 to 18×10^9/l (day 3), recovered on day 7. Furthermore a strong reticulocytosis occurred on day 14 (27%). The level of Elastase-1AT complexes rose slightly (2–24 h) and the plasma tPA also increased. There were no deviations in the PTT, APTT, fibrinogen, TAT complexes, C4bc or C3bc.

The other 4 monkeys of the PolyHbXl group did not show any clinical symptoms. However, in two monkeys several small petechiae were observed on the skin. One of the four monkeys had a reversible thrombocytopenia starting after day 1; from 378 until 68×10^9 on day 3, recovered on day 7.

The antibody response. In all monkeys antibodies against both HSA and (human) PolyHbXl were detected at 14 d after the infusion. Before the infusion no antibodies were detectable. No neo-epitopes related to the preparation proces were detected.

Discussion. General. The strong hypotensive reaction after withdrawal of the blood at 10 ml/kg was probably directly due to the anesthesia. The acute phase reaction observed in all monkeys appears to be inherent in the model and indicates tissue ischemia during the hypotensive period.

The monkey with kidney insufficiency and thrombocytopenia. The renal insufficiency in this animal was probably mainly caused by the tissue ischemia that occurred during the phase of hypotension. In this monkey the mean blood pressure decreased from 153 to 40 mmHg (during about 15 min). Hardly any hemoglobin could be detected in the urine (in the first 2 h 40 mg of the infused dose of 14000 mg). Therefore, it is not probable that the toxicity was caused by leakage of the Hb through and/or accumulation of it in the tubuli. A reasonable possibility is that PolyHb has caused vasoconstriction in the kidneys aggrevating the ischemia. After 5 h thrombocytopenia was observed that worsened gradually during 3 d. This time course makes it unlikely that the thrombocytopenia was caused by an immune reaction. The anal bleeding and reticlocytosis strongly suggest that internal bleeding caused an increased

consumption. There were no indications of coagulopathy. We hypothesize therefore that the bleeding disorder may have been caused by endothelial cell damage.

Conclusion. The results point to a "stressed model" with a period of hypotension and induction phase reaction. PolyHbXl caused: (1) in 1 of the 5 monkeys a reversible acute renal insufficiency and a reversible, severe thrombocytopenia, probably due to a bleeding disorder; and (2) no serious abnormalities in the 4 other monkeys.

Histopathology in the rat

Purpose. The purpose of the investigation was to examin macroscopic and microscopic changes over a period of 15 d after Hb infusion. In earlier investigations, uptake of unmodified Hb was observed in liver and kidney cells. Especially in tubular cells high concentrations of Hb were found. Therefore we not only studied general morphological changes, but also the uptake in liver cells, spleen cells and tubular epithelial cells as well as accumulation in the tubular lumina. Fourty-two rats were subjected to an exchange transfusion in which of 20 ml/kg of blood were withdrawn, followed by the infusion of 40 ml/kg of PolyHbXl ($n = 16$) or of human albumin (HSA, $n = 16$) as a control solution. As sham operated controls, 6 rats were used. The rats were observed and sacrificed for necropsy at predetermined time points: after 1, 5, 24, 48 h, 8 or 15 d. Liver, spleen, kidney and organs with macroscopic changes were examined by light microscopy. Hb deposition was shown by diaminobenzidine staining.

Results. None of the rats showed any sign of morbidity. With regard to the blood cell counts there were no differences between the PolyHbXl- and the HSA-treated groups. After the exchange transfusions the hematocrit (Hct) values decreased by about 50%, but returned to normal values after 8 d. In the first week the reticulocyte count was increased and there was also granulocytosis. After 1–2 d the platelet count was increased in some rats. None of the rats showed a decrease in platelet count.

There was no difference in body weight and organ weight between the animals of the two groups and the urine production and fluid balance were also equal. After 48 h, 2 of the 18 rats treated with PolyHbXl showed haemorrhagic spots in the wall of the jejunum. At these spots both extravasation of red cells into the mucous and muscle layer and infiltration with granulocytes was observed. In the liver the hepatocytes showed many vacuoles between 1 and 24 h with a maximum at 5 h.

After 24 h the vacuoles had disappeared. In the Kupfer cells a pronounced uptake of Hb was observed, but at a later stage: the maximal uptake was after 24–48 h and after 8 d it had disappeared. In the kidney, after 5 h minimal uptake was observed in epithelial cells of the proximal tubuli and also in glomerular macrophages.

Conclusions. (1) The modification of Hb to PolyHbXl prevents leakage through and accumulation of Hb in the tubules of the kidney. Only a minimal amount of this preparation was secreted by the kidney. There was no signs of damage to the kidney.

(2) PolyHbXl was taken up and metabolized by the hepatocytes and Kupfer cells in the liver but there were no indications that these cells were damaged. (3) In 2 out of 3 rats autopsied after 48 h, some hemorrhagic lesions were observed in the intestinal wall. Because we also found evidence of intestinal bleeding in the monkey model as well as in the rabbit model, we decided to use the rat model to analyze this hemorrhagic disorder.

Analysis of the hemorrhagic disorder

Model. We analyzed the above mentioned hemorrhagic side effect in a similar histopathological study in rats. There was a minor difference in that a permanent cannula was implanted for blood sampling after the infusions. Full details of this model and the complete analysis are described elsewhere [17]. The main conclusions were:

1. By comparing different Hb solutions and subfractions we found that the transient hemorrhagic disorder was due to the glutaraldehyde treatment we used in the preparation of PolyHbXl.
2. This disorder was relatively mild and the lesions became manifest after more than one day and were resolved in about one week. The effects may easily be overlooked in routine preclinical safety testing.
3. The stressed animal model used in this study is sensitive in detecting this toxic effect.
4. The hemorrhagic lesions appeared not to be due to thrombocytopenia or depletion of coagulation factors. The lesions have the character of a "small vessel vasculitis". The hypothesis is that endothelial cell damage plays a central role in the pathophysiology.

Summary and discussion

Blood pressure, haemodynamics and PolyHbXl

The rat as well as rabbit models show a rise in blood pressure (BP) after infusion of PolyHbXl. This effect is generally explained by the hypothesis that this rise in BP is mediated mainly by the binding of NO by the Hb molecules themselves, while NO functions as EDRF (endothelial derived relaxation factor). The observation that the polymer fraction of PolyHbXl still produces a rise in blood pressure does not support the assumption that 64 kD Hb molecules are the only molecules in a product like PolyHbXl which influence the BP.

In the pig model we did not observe systemic hypertension. Interestingly, the blood flow distribution between various organs was not affected. This may take away the concern for disturbance of the regional flow distribution by Hb solutions in vivo. However, this is with the exception of the blood flow from the LV through the lungs which was decreased. Further, there was an increased pulmonary arterial blood pressure. The latter observations should be taken into account, particularly

when infusion in patients in whom lung function problems can be expected, is considered.

The systemic oxygen consumption of the hypovolemic pigs infused with PolyHbXl is equal to the that of the autologous blood-treated pigs. As far as we have analyzed the data now the lower oxygen saturation and oxygen pressure in the circulation does not point to a disadvantage for the function in the PolyHbXl-treated pigs.

Nephrotoxicity and PolyHbXl

From the observations with the rat kidney model it could be concluded that (1) a purified and unmodified Hb solution impairs renal function after hemorrhage and shock. Whether this is due to obstruction of the kidney tubuliby the leaking $\alpha\beta$ dimers is not clear. In a euvolemic model (the same model without a period of shock) no damage was observed after infusion of SFHb. (2) the acceptable level of contamination of PolyHbXl with non-crosslinked Hb is about 2–3% of the total Hb content. This is based on the observation that PolyHbXl, in contrast with non-modified SFHb, did not cause any damage to the kidney in the hypovolemic model. The tested preparation contained 2–3% non-crosslinked (dissociable) Hb. The pig and rabbit didn't show signs of renal toxicity by PolyHbXl either. From the histopathological rat model it could be concluded that the modification of Hb to PolyHbXl prevents leakage through and accumulation of Hb in the tubules of the kidney. Only a minimal amount of this preparation was secreted by the kidney. There were no signs of damage to the kidney.

Thrombogenicity and PolyHbXl

A guinea-pig model had been developed in our laboratory in order to assess the thrombogenic risks of Hb solutions [16]. We used a non-stasis model in which we follow the generation of fibrinopeptide A, one of the most sensitive markers for the activation of thrombin in vivo. The sensitivity of the model was increased by infusion of a small dose of factor Xa. PolyHbXl and SFHb were tested with HSA as a control solution. No activation was detected after infusion of PolyHbXl or HSA. Only in the SFHb group a small rise of FPA was found: 5 ng/ml (control level, <2 ng/ml). This rise is probably due to the presence of a small amount of phospholipid in this solution. The batch of PolyHbXl was not thrombogenic.

Also from earlier data it can be concluded that properly purified Hb solutions (with minimal phospholipid contamination) do not activate the coagulation system. In general it can be stated that proper purification and modifcation are essential requirements for the safety of hemoglobin solutions for clinical use.

The hemorrhagic side effect of PolyHbXl

In the monkey, rabbit and rat model we observed in some animals indications for a hemorrhagic disorder. In the monkey model, which was a "stressed model" with a

period of hypotension and induction of an acute phase reaction, PolyHbXl caused in 1 of the 5 monkeys a reversible, severe thrombocytopenia and signs of intestinal bleeding, which was probably the cause of the thrombocytopenia. A less severe thrombocytopenia was also observed in a second monkey.

In the rabbit study 1 out of 6 animals showed thrombocytopenia and died after four days with intestinal bleeding.

In the histopathological rat model in 2 out of 3 rats autopsied after 48 h, some hemorrhagic lesions were observed in the intestinal wall. Although the side effect was mild and transient (not found in the rats autopsied at other time points), we decided to use the rat model to analyze this hemorrhagic disorder with the hypothesis that the hemorrhagic disorders observed in the different models are based on the same pathological mechanism. The main conclusions were:

1. By comparing different Hb solutions and subfractions we found that the transient hemorrhagic disorder was due to the glutaraldehyde treatment we used in the preparation of PolyHbXl. Another similar product, Hemolink, which is polymerized with a ring-opened sugar by Hemosol, did not cause this disorder.
2. This disorder was relatively mild and the lesions became manifest after more than one day and were resolved in about one week. The effects may easily be overlooked in routine preclinical safety testing.
3. The stressed animal model used in this study is sensitive in detecting this toxic effect.
4. The hemorrhagic lesions appeared not to be due to thrombocytopenia or depletion of coagulation factors. The lesions have the character of a "small vessel vasculitis". The hypothesis is that endothelial cell damage plays a central role in the pathophysiology.

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Blood Substitutes — Present and Future Perspectives
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CHAPTER 19

The Heme Oxygenase System In Liver Microcirculation: A Key Mechanism for Hemoglobin Degradation

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Introduction

Modified hemoglobin derivatives have been developed as blood substitutes clinically applicable to rescue shock states. However, their degrading process in reticuloendothelial system and its pathological consequences remain unknown. Physiological degradation of heme (iron-protoporphyrin IX) into biliverdin, iron and carbon monoxide (CO) is mediated by heme oxygenase (HO), which consists of two distinct isoenzymes called HO-1 [1] and HO-2 [2]. HO-1, also known as the heat shock protein-32 (hsp 32), is induced by a variety of stressors such as hyperthermia [3], cytokines [4], and intake of heavy metals [5]. This isoform is considered to be present in spleen, a major organ for destruction of senescent erythrocytes, and in liver stimulated with endotoxin [6] or ischemia-reperfusion [7]. On the other hand, HO-2 is a constitutive form and is known to be abundant in brain, testis and unstimulated liver of rodents and human, as reported previously [8]. Because of little information as to topographic distribution of the heme oxygenase system, a fate of artificial blood substitutes administered in the body has not fully been investigated at cellular and molecular mechanisms in vivo. This chapter aimed to focus on biological significance of distribution of HO isoforms in hepatic microvascular units as a primary determinant of heme degradation.

Heme oxygenase-1: a primary anti-oxidative defense mechanism

Heme is metabolized to biliverdin and CO by heme oxygenase (Fig. 1). In humans, nearly 80% of the bilirubin excreted in bile is derived from hemoglobin heme [2]. Cytochrome P450 is a major contributor to the bilirubin derived from non-hemoglobin sources. Recent investigation has revealed that the inducible form of heme oxygenase (HO-1) plays an important role in modulation of cell susceptibility to oxidative stress or lipid peroxidation. Namely, the induction of this enzyme can

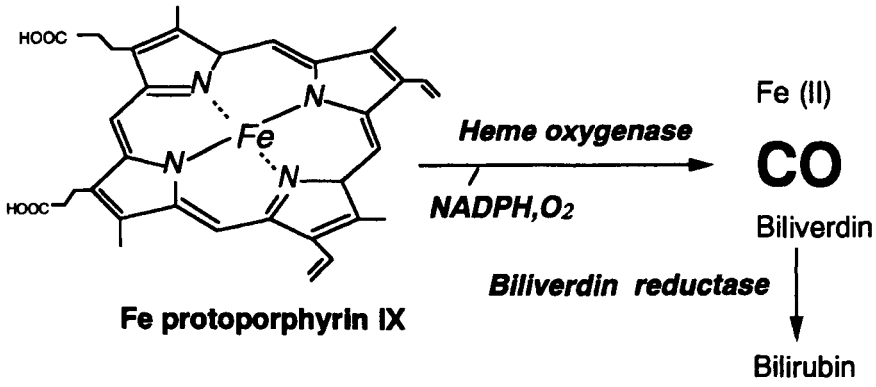

Fig. 1. Heme oxygenase and generation of carbon monoxide and bilirubin.

reduce the intracellular free heme pool and increase bilirubin. Furthermore, the heme oxygenase reaction is known to regulate the expression of ferritin, the iron-storaging protein through the mechanism involving free iron yielded by heme degradation. When skin fibroblasts are exposed to ultraviolet (UV) A, these cells induce ferritin and acquire resistance to the second exposure to the lethal dose of UVA. The UV-induced induction of ferritin and acquisition of anti-oxidant properties can be abolished by inhibiting heme oxygenase or by chelating the iron by desferrioxamine. These results imply the importance of HO-1 as a crutial enzymatic intermediate in an oxidant stress-inducible antioxidant defense mechanism involving the induction of ferritin [9].

Another possibility by which the HO reaction serves as an anti-oxidative defense mechanism can be explained by its involvement in generation of bilirubin, a potent endogenous radical scavenger [10,11]. Bilirubin is generated by the reaction of biliverdin reductase, a terminal step for heme degradation. Because of its capability to scavenge single oxygen and hydroxyl radicals, bilirubin is called "suicide anti-oxidant". It has well been demonstrated that bilirubin endogenously upregulated as a consequence of an induction of HO-1 plays a role in reducing the oxidative impact under disease conditions such as endotoxemia. It has also been shown that bilirubin can attenuate transendothelial migration of macrophages prestimulated with oxidized low-density lipoprotein.

Roles of carbon monoxide derived from heme oxygenase in the liver

The HO reaction generates another biologically important product, that is, CO. Can this gaseous monoxide exert its biological actions to regulate cell and organ function? Since the putative signal transducing mechanism of CO and NO involving soluble guanylate cyclase-cGMP pathway was recently proposed, several lines of

circumstantial evidence have emerged illustrating the mechanisms for CO-dependent regulation of cell function. Such evidence was first raised by Snyder and his coworkers, who showed that endogenous CO suppression by zinc protoporphyrin IX (ZnPP), a competitive inhibitor of the HO reaction, induces reduction of cGMP in olfactory nerve cells which possess little NO synthase in spite of the presence of soluble guanylate cyclase [12]. As shown later, we reported that ZnPP administration to the perfused rat liver raises an increase in the vascular resistance which can be recovered by supplement with micromolar levels of CO [13–15]. In addition, it has recently been shown that the inducible form of heme oxygenase is expressed by vascular smooth muscle cells and CO derived from the enzyme activity determines cGMP levels in these cells, suggesting that the possible role of endogenous CO in autocrine regulation of vascular tone [16].

On the other hand, evidence has recently been provided that, as compared with NO, CO is not such a potent mediator that it cannot activate soluble guanylate cyclase. The ability of NO to activate this enzyme reaction is approximately 50-fold greater than that of CO [17]. Different from NO, CO is a non-radical monoxide, and its reactivity with biological reagents which can interact with NO such as SH compounds or non-heme iron is far less than that of NO. In other words, CO cannot function as a potent activator of guanylate cyclase under circumstances in which the local concentration of NO is at relatively higher levels than that of CO. It is therefore impossible to demonstrate the significance of CO in cGMP-dependent regulation of cell and organ function without discussing the actual amounts of the gaseous mediator in situ and the relationship of microanatomical orientation between the CO-generating effector cells and target cells in each experimental system.

Distribution of heme oxygenase isoforms in liver

Liver is a major organ in the body which continuously generates CO through the HO reaction. Several lines of evidence have indicated that the HO reaction serves as a key mechanism to maintain the integrity of physiological function of organs such as liver. As described previously, zinc protoporphyrin IX (ZnPP), a heme oxygenase inhibitor, elicits a marked increase in the vascular resistance as a consequence of sinusoidal constriction [13,14]. Furthermore, the ZnPP administration turned out to induce bile acid-dependent choleresis which coincided with a depletion of the venous CO flux and biliary excretion of bilirubin, another product of heme degradation [18]. The results have thus suggested that CO generated by heme oxygenase serves as an endogenous regulator of hepatobiliary functions under physiological conditions.

In an attempt to understand the aforementioned roles of heme oxygenase, we have recently investigated the intrahepatic distribution of HO-1 and HO-2 using newly developed monoclonal antibodies (MoAbs) against these isozymes [19]. The immunohistochemical analysis disclosed different topographic patterns in the distribution of the two isozymes. As seen in the left panel of Fig. 2, HO-1 distributed in a relatively

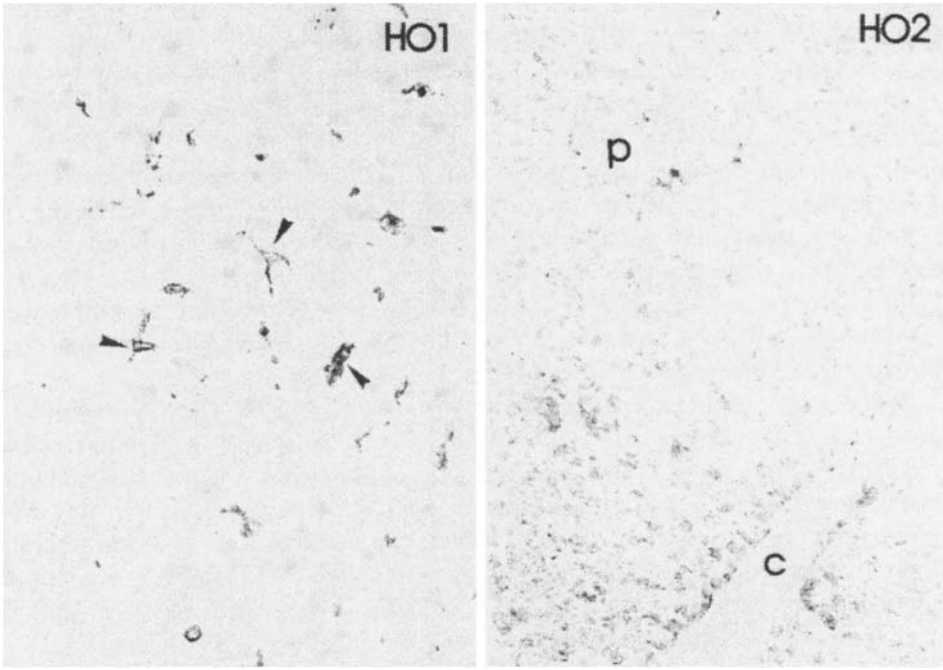

Fig. 2. Expression of HO-1 and HO-2 in the normal rat liver. The 10-μm thick sections were immunostained with MoAbs against HO-1 (left) and HO-2 (right). P and C denote portal and central venules, respectively. Arrowheads suggest that Kupffer cells constitute a major site of the HO-1 expression. Bars represent 50 μm and 100 μm in left and right panels, respectively. Cited from J. Clin. Invest. vol 101, 1998 with permission.

small number of cells which scattered over the entire lobules. These HO-1-positive cells were characterized by their irregular and dendritic shapes and by protrusion of their cytoplasm towards sinusoidal spaces, suggesting the presence of HO-1 in Kupffer cells. On the other hand, hepatocytes exhibited little staining, if any. By contrast, HO-2 occurred in the parenchymal cells and distributed homogeneously among the entire lobules, while nonparenchymal cells displayed practically no staining (right panel in Fig. 2). We further attempted to determine specific cell types that express HO-1 among the nonparenchymal cells, indicating that hepatic stellate cells exhibited little HO-1 staining, if any. These findings indicate that Kupffer cells constitute a major cellular compartment responsible for the intrahepatic HO-1 expression.

The intrahepatic distribution of HO-1 exhibited quite different pictures when the liver was prestimulated with LPS, an inducer of this enzyme [19]. The liver exposed to the 6-h pretreatment revealed that HO-1 not only occurred in tissue macrophages but was also induced markedly in hepatocytes. On the other hand, the HO-2 staining did not display any notable changes as compared with the control liver. These results

indicate that Kupffer cells and hepatocytes constitute major cellular components that express the inducible HO isozyme in the endotoxin-treated liver.

Microtopographic basis for CO-mediated vasorelaxation in liver

Blood substitutes which are going to be applied in the clinical field can not only bind molecular oxygen but also trap vasodilating gaseous monoxides such as NO and CO. Considering that endothelial cells in the hepatic microvessels (sinusoids) possess abundantly small pores called sieve pores or fenestration which allows macromolecules in circulation to access the extrasinusoidal space (the space of Disse) (Fig. 3), it is hypothesized that hepatic microvascular responses may differ between stroma-free hemoglobin (Hb) molecules and liposome-encapsulated Hb (HbV) that cannot access the space of Disse because of its greater size than that of fenestration. We have attempted to examine this hypothesis in isolated perfused liver preparation. As seen in Fig. 4, immediately after the start of HbO_2 administration at a concentration of 1.5 g/dl, the resistance increased markedly, showing a 25% elevation as compared to that in the steady-state conditions. When the perfusate was replaced by the Hb-free buffer at 15 min, the increased resistance decreased gradually and reached the control level at 30 min, indicating that the HbO_2-induced vascular response is reversible. It

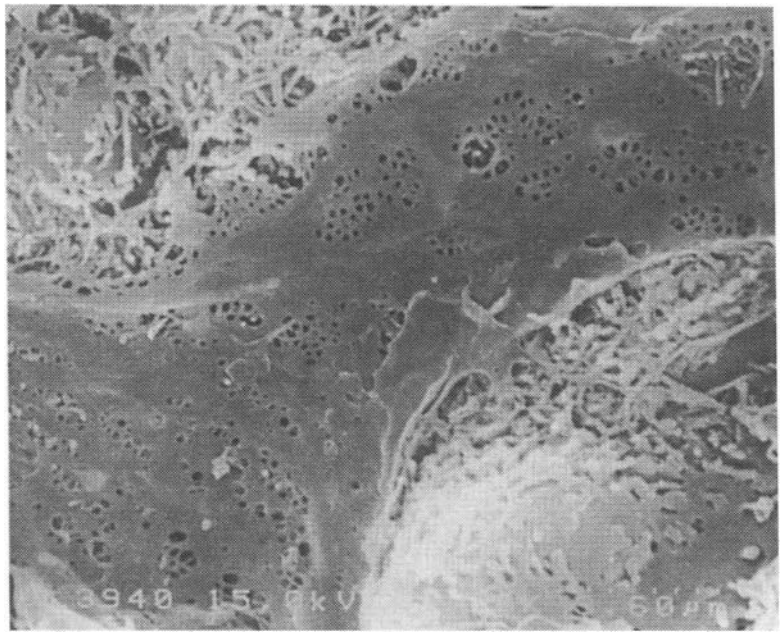

Fig. 3. A representative scanning electron micrograph showing sieve pores in sinusoidal endothelial cells in the rat liver. Size of pores are in a range between 100–150 nm in diameter.

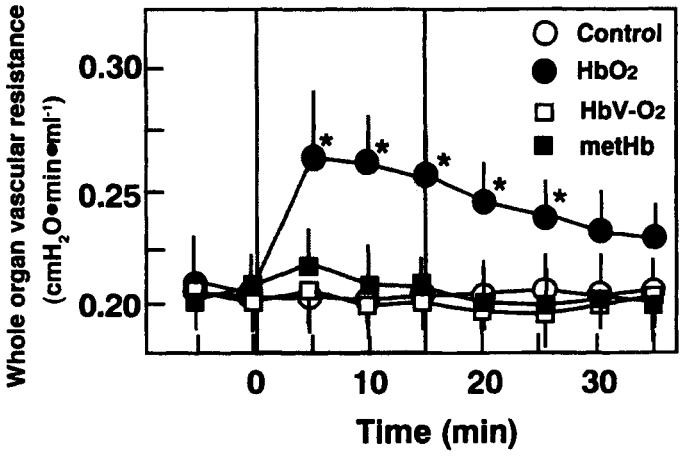

Fig. 4. Time history showing effects of Hb derivatives on the whole organ vascular resistance in perfused rat liver. The Hb derivatives (HbO_2, HbV-O2, metHb) were administered at a final Hb concentration of 1.5 g/dl for 15 min as seen in the shaded area. Data represent mean ± SE of 6 experiments in each groups. Open and closed circles indicate the data collected from the control and HbO_2-treated livers, respectively. Shaded circles and open squares are those collected from the groups treated with HbV-O_2 and metHb, respectively. $*P < 0.05$ as compared with the data in the control group. Cited from J. Clin. Invest. vol 101, 1998 with permission.

should be noted that the HbV-O_2 administration did not evoke any significant elevation in the resistance. These results suggest that the HbO_2-induced elevation of the vascular resistance was abolished when the diffusion of Hb across the sinusoidal endothelium into the space of Disse was blocked by its liposomal encapsulation. In addition to these findings, metHb, a reagent that could scavenge NO but not CO, did not alter the vascular resistance, suggesting that CO plays a major role in lowering the vascular resistance under the current experimental conditions. Furthermore, under these experimental conditions, inhibitors of NO synthase such as *N*-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine did not alter the basal vascular resistance. These results suggest that involvement of NO is little, if any. Taking these circumstances into account, we suggest that, once administered, stroma-free HbO_2 diffusing into the space of Disse eliminates CO in situ and thereby evoking sinusoidal constriction.

Design of blood substitutes based upon heme oxygenase biology

When the previously proposed mechanisms for intrahepatic heme degradation are taken into accounts, the present findings suggest a possible cooperative role of these two isoforms in catabolism of hemoglobin-derived heme in different cellular

compartments. Namely, previous studies by Bissel et al. [20] revealed that removal of senescent erythrocytes from the circulation is carried out by macrophages in the liver and spleen, while hemoglobin released as a consequence of erythrocyte destruction can be metabolized mainly in the liver parenchyma. Spontaneous expression of the inducible HO isoform in Kupffer cells of the control liver appears to result from constant exposure of the cells to senescent erythrocytes in the sinusoidal compartment, inasmuch as such an expression of HO-1 is evident as well in macrophages in red pulp of spleen, another major compartment for the erythrocyte removal and heme degradation [2]. On the other hand, the liver parenchyma is considered to be a major cellular compartment for localization of non-hemoglobin heme proteins such as cytochrome P450 [21]. Since heme molecules of these enzymes are known to be metabolized almost exclusively by the heme oxygenase reaction [2], it is not unreasonable to suggest that, in the normal liver, HO-2 limits intrahepatic turnover of the heme enzymes.

Considering a compartmentalization of CO generation characterized by intra- and extra-sinusoidal distribution of specific HO isoforms, these lines of knowledge on heme degradation could provide a clue to refine the ideal design of blood substitutes. Intra- or intermolecular crosslinking is thought to consider a potential strategy to prevent renal ultrafiltration end to elongate the halflife of blood substitutes. In organs possessing fenestrated endothelial cells such as the liver, however, sieve pores could allow stroma-free hemoglobin molecules to access the space of Disse and scavenge the vasodilating gaseous monoxides. Furthermore, the stroma-free hemoglobins are unlikely to be catabolized in the HO system in Kupffer cells, where heme molecules derived from scenecent erythrocytes are mainly degraded, but would be degraded in the parenchymal cells. Such circumstances might jeopardize hepatocytes against oxidative stress and cause unnecessary expression of the inducible HO system and CO generation. Crosslinking of hemoglobins could delay a physiologic turnover of heme degradation by the HO system and, once administered, would stay in the extrasinusoidal space as a scavenger of the gaseous vasodilators. These possibilities should carefully be examined in the liver under normal and disease conditions such as endotoxemia or hemorrhagic shock, where HO-1 or NO synthase are overexpressed prior to the administration of the Hb derivatives. However, the present study suggest that liposomal encapsulation of the Hb derivatives serves as a potential strategy to reduce these risks to perturb the hepatic microvascular function.

Acknowledgments

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Blood Substitutes — Present and Future Perspectives
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CHAPTER 20

Vascular Activities of Hemoglobin-Based Oxygen Carriers

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Introduction

The major concerns in the development of hemoglobin (Hb)-based oxygen carriers (HBOCs) have been the modulation of increased oxygen affinity and the short circulatory retention of native Hb. These problems have been resolved successfully by strategic chemical and genetic modifications of the protein, and clinical trials using some acellular Hb derivatives have already started (reviewed in Tsuchida) [1]. Such modification methods include intramolecular and intermolecular crosslinking, conjugation to an inert material such as polyethylene glycol (PEG), and polymerization. However, it has been recently shown that the infusion of acellular Hb derivatives causes hypertension [2–4], gastrointestinal symptoms [5,6], and platelet activation [7,8]. In this report, we focus on the vascular effects of HBOCs.

Historical overview

One of the early reports that noted Hb-related hypertension was by Bayliss [9] in 1920. He treated shocked animals with a small amount of filtered hemolysate, and observed a slight increase in blood pressure and an increase in urinary output. Amberson et al. [10] also observed hypertension and bradycardia in patients after infusion of a filtered hemolysate. Miller and McDonald [11] reported that nephrotoxic effects after the infusion of a crude Hb solution were caused by renal vasoconstriction-induced ischemic reaction.

From these early experiments, the basic question arose as to whether Hb itself is toxic. In 1967, Rabiner et al. [12] successfully infused stroma-free Hb (SFH) into animals without severe nephrotoxicity. This suggested that contamination such as stroma was responsible for Hb-related toxic reactions. However, Savitsky et al. [13] reported that SFH still induced abdominal pain, costovertebral angle tenderness, bradycardia, mild hypertension, decreases in urine output and endogenous creatinine clearance, and inhibition of blood coagulation. Focusing on the cardiovascular effects, Vogel et al. [14] provided confirmative data indicating the vasoconstrictive

activity of SFH in isolated rabbit hearts. They also reported that polymerized pyridoxalated Hb still had a reduced vasoconstrictive activity.

In the 1990s, the vascular activity of Hb derivatives has been reevaluated using highly purified SFHs since the conventional SFHs still contain a detectable amount of stroma [15]. The results showed that the hypertensive effect was closely related to the modification method used to produce the derivative; intramolecularly crosslinked Hb such as DCLHb increased blood pressure by 20–30 mmHg [2–4], while PEG-conjugated Hb [16] and polymerized Hb [17–19] had reduced or no vasoactivity.

Background: EDRF and vasoconstriction

It is not surprising that acellular Hb derivatives have vasoconstrictive activity. Endothelium-derived relaxing factor (EDRF) has recently been identified as nitric oxide (NO) or an NO-containing moiety [20], and Hb binds NO in two ways; the heme iron of Hb irreversibly binds NO with an extremely great affinity. The sulfhydryl groups of cysteine of the protein also form a stable complex with NO [21]. This suggests that Hb itself is the major factor responsible for the observed vasoconstriction.

However, some puzzles still remain. The reason why a very small amount of acellular Hb induces significant vasoconstriction is unclear because peripheral blood already contains a large amount of cellular Hb in red cells. It seems unlikely that acellular Hb-induced vasoconstriction is produced solely in the same way that red cells inhibit EDRF. Furthermore, the molecular mass of acellular Hb seems to have a significant impact on its vasoconstrictive activity because PEG-modification significantly reduced the vasoconstrictive property of DCLHb [22]. The encapsulation of Hb into liposomes also reduced vasoconstriction in a coronary perfusion experiment [23] and in vivo [24]. It is clear that a more detailed understanding of the underlying mechanism(s) by which Hb induces vasoconstriction is necessary to ensure the safety of HBOCs. We have performed several in vitro experiments in an attempt to achieve this goal.

Organ bath experiments

The preparation of human and bovine SFHs and several acellular Hb derivatives were described previously [25]. PEG-modified pyridoxalated human Hb (PEG-Hb) was a generous gift from Dr Y. Iwashita (Ajinomoto Co. Ltd., Kawasaki, Japan). Intramolecularly crosslinked Hb (XL-Hb) was prepared from human SFH with bis(sulfosuccinimidyl)suberate [26]. Two liposome-Hb preparations, a liposome stabilized by membrane phospholipid polymerization (ARC) [27] and a PEG-modified liposome (NRC)[28], were provided by NOF Company (Tsukuba, Japan)

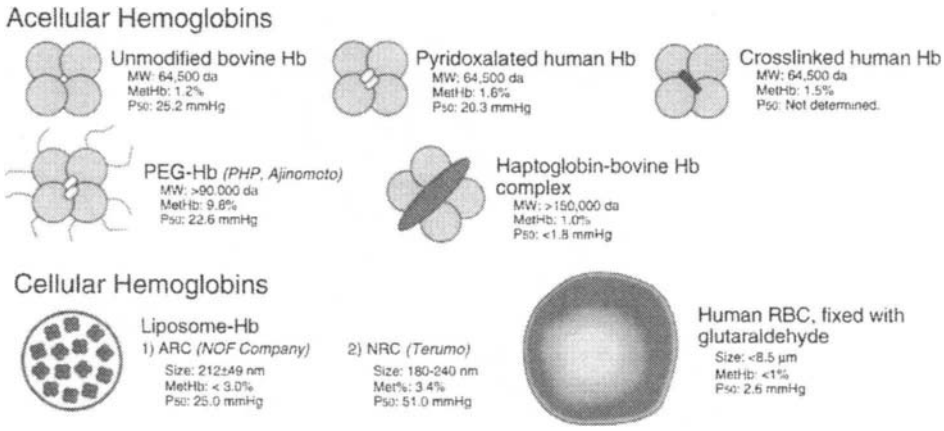

Fig. 1. Physicochemical characteristics of Hb preparations used in this report.

and Terumo (Kanagawa, Japan), respectively. Their physicochemical characteristics are illustrated in Fig. 1.

We have characterized Hb-induced vasoconstriction in an organ bath system using rabbit aortic strips [29,30]. In this in vitro experiment, all Hb preparations reversed acetylcholine (ACh)-induced relaxation in a dose-dependent manner. These observations were already reported by others [31,32]. On the other hand, we also noticed an interesting phenomenon in that the repeated exposure of tissues to unmodified Hb gradually reduced the responsiveness to ACh. We therefore performed an experiment: the tissues were pretreated with several Hb preparations for 30 min and then ACh-induced relaxation was recorded in the absence of Hb. Exposure to bovine SFH induced a significant reduction of the following ACh-induced relaxation, while the relaxation was either marginally reduced or not affected by Hp-Hb, PEG-Hb, liposome-Hb (ARC) and glutaraldehyde-fixed RBCs (Figs. 2 and 3). This persistent inhibitory effect by unmodified Hb disappeared when the tissue was further incubated for 2–3 h with changed medium, indicating the reversibility of this inhibitory action and the functional integrity of the endothelium. Although vasoconstrictive factor(s) other than Hb might have contributed somewhat, this would not be expected to cause a significant effect, since Hb-Hp also contained the same components as unmodified Hb.

We performed similar experiments in the presence of 2% BSA or 2% dextran (mean MW 60,000–90,000 D). As shown in Fig. 3, both macromolecules partially prevented Hb-induced vasoconstriction.

These observations led us to propose the hypothesis that Hb-induced vasoconstriction needs infiltration of Hb into the tissues, probably through the intercellular cleft of the endothelium. It is likely that macromolecules such as albumin and dextran compete with Hb for access to the tissue.

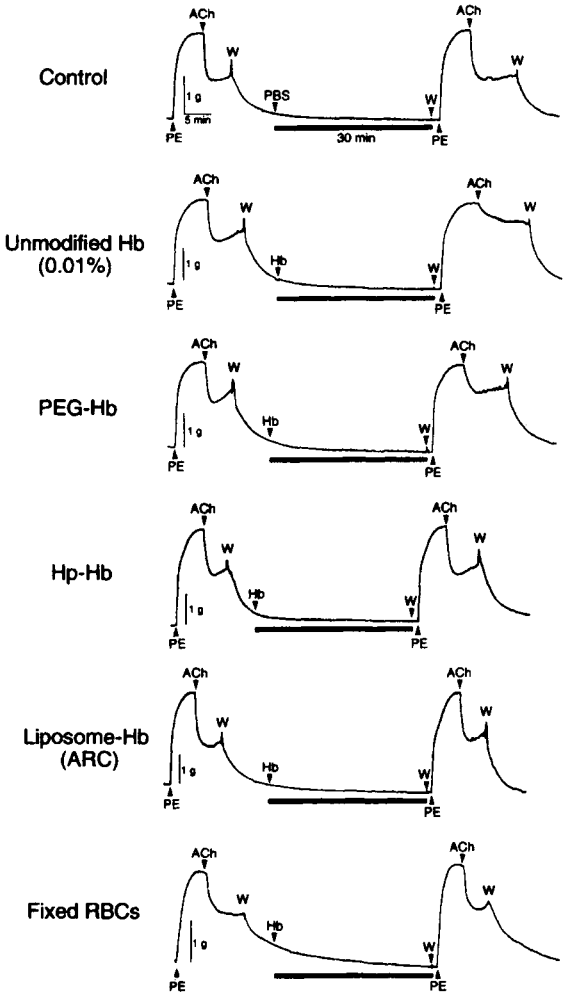

Fig. 2. Typical tracings showing inhibition of ACh-induced relaxation by several Hb preparations (Hb at 0.01%) in the organ bath assays using rabbit aortic strips. PE, phenylephrine 1 μM; ACh, acetylcholine 1 μM; W, wash-out.

Heart perfusion experiments

We have also examined the vascular activity of Hb preparations in a Langendorff perfusion model of rat hearts [23]. Changes in perfusion pressure and endothelium-dependent vasorelaxation, which were induced by a bolus injection of bradykinin (BK), were monitored.

Perfusion of rat hearts with bovine SFH and PEG-Hb increased perfusion pressure in a dose-dependent manner, and typical tracings are shown in Fig. 4. Unmodified

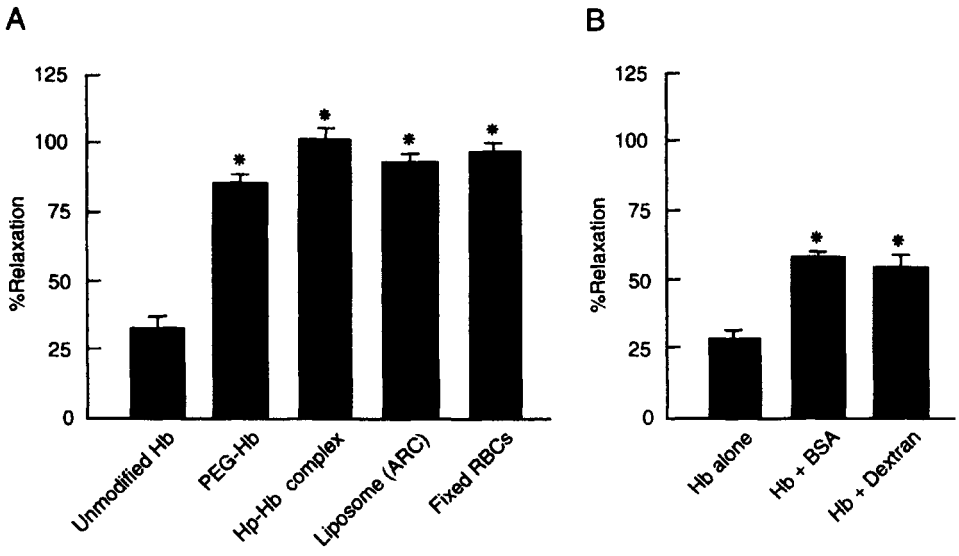

Fig. 3. Inhibition of ACh-induced relaxation by Hbs. A, Inhibitory effect of several Hb preparations (Hb at 0.01%). B, Unmodified Hb-induced inhibition in the presence of 2% BSA or 2% dextran. Means ± SE of 5–12 experiments. $*P < 0.01$ as compared with unmodified Hb.

bovine Hb increased coronary perfusion pressure and decreased the duration of BK-induced relaxation (Fig. 5). PEG-Hb also presented a similar vasoconstrictive profile with a reduced extent. In contrast, liposome-Hb (NRC) did not cause any vasoconstrictive response. We also observed that another liposome-Hb, ARC, had no vasoconstrictive activity [23].

Both SFH and PEG-Hb affected only the duration of BK-induced relaxation, while neither none of them affected the amplitude of the relaxation. These vasoconstrictive profiles of acellular Hbs were in good agreement with the report of Sakuma et al. [33] who showed Hb-induced inhibitory effects on endothelin-induced vasodilation. BK-induced relaxation is caused by a combination of at least two factors, EDRF and endothelium-derived hyperpolarizing factor (EDHF); the duration of BK-induced relaxation might be mainly related to EDRF, while the amplitude of the relaxation might be related to EDHF. The profiles of BK-induced relaxation in the presence of Hb suggested that Hb disturbed only the action of EDRF. This point was also confirmed by the addition of a specific inhibitor for NO synthase, NAME, to the perfusate. NAME induced further vasoconstrictive effects in the Hb-induced response, but the extent of changes was identical for all conditions.

We also performed this perfusion experiment in the presence of BSA (0–2%) in the perfusate. BSA significantly delayed the acellular Hb-induced vasoconstrictive response in a dose-dependent fashion, while the extent of increase in perfusion

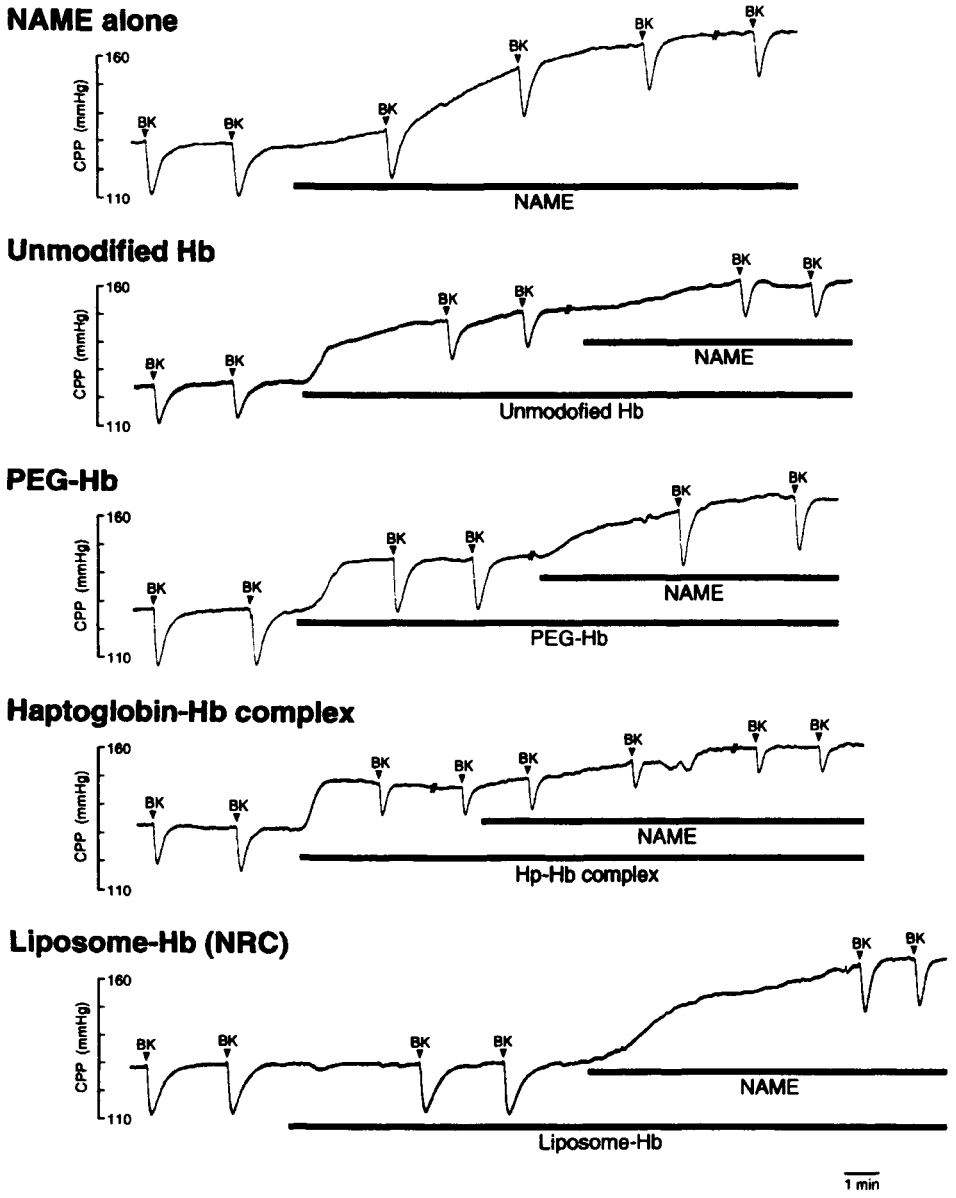

Fig. 4. Typical tracings showing Hb (0.1%)-induced vasoconstriction in rat perfused hearts. Hearts prepared from male Wister rats were perfused with Krebs-Henseleit solution containing 10 nM U-46619 and 0.2% BSA. Perfusion flow rate was adjusted as to maintain the coronary perfusion pressure (CPP) at 120–140 mmHg. BK, bradykinin 10 pmol in 4 μl; NAME, N^{ω}-nitro-L-arginine methyester 100 μM.

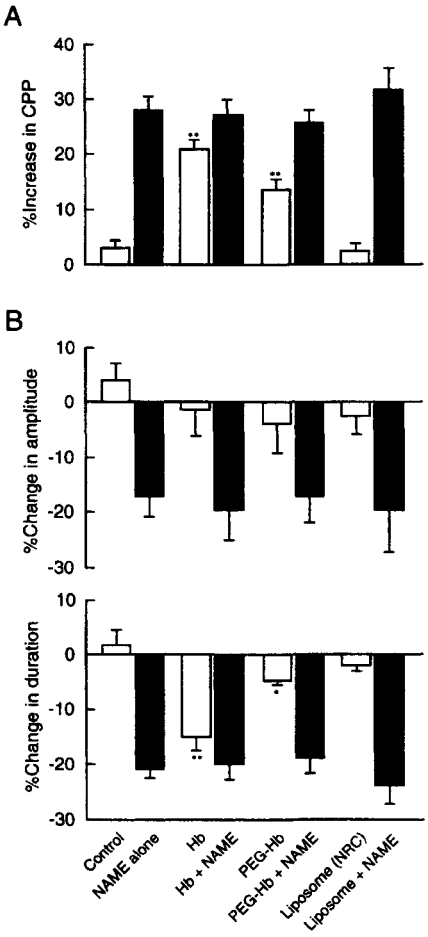

Fig. 5. Effects of Hb preparations on CPP and BK-induced relaxation. A, Changes in CPP. B, Changes in amplitude and duration of BK-induced relaxation. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with control. Mean ± SE of 8–10 experiments.

pressure attained was not affected. Macromolecules such as BSA may inhibit Hb extravasation competitively.

Endothelium permeability experiments

Endothelial permeability characteristics of Hb preparations should be closely related to their vasoconstrictive activities if Hb induces vasoconstriction as a result of abluminal EDRF scavenging. Thus, we measured the transendothelial fluxes of several Hb preparations using a bovine thoracic aortic endothelial cell monolayer cultured on a microporous membrane as shown in Fig. 6 [34]. With medium placed in

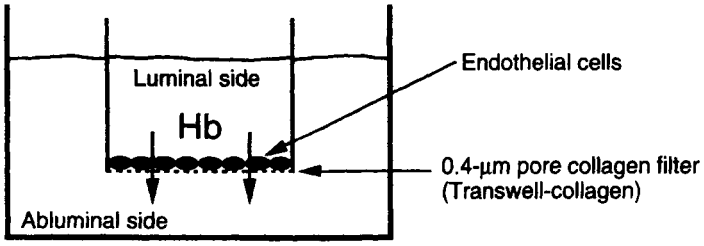

Fig. 6. Permeability measurement using cultured bovine endothelial cell monolayer. Bovine thoracic aortic endothelial cells were grown to confluence on culture plate inserts with a porous collagen filter.

both the inner and outer wells, two chambers that were separated by the endothelial monolayer grown on the collagen filter were created. The data obtained were expressed in terms of the permeability coefficient (expressed in cm/s) [35]. The calculated permeability coefficient of BSA in this system was comparable to those of previous reports [35,36].

Figure 7 shows the permeability characteristics of Hb derivatives. With the untreated monolayer, the permeability coefficient of unmodified Hb was almost twice that of BSA. Intramolecular crosslinking slightly but significantly decreased the permeability of Hb. Other Hb modifications such as PEG modification and Hp binding profoundly reduced the transendothelial flux of Hb. Liposome-Hb (ARC) showed an even lower permeability coefficient. These results demonstrated for the first time that Hb derivatives having smaller molecular masses had increased

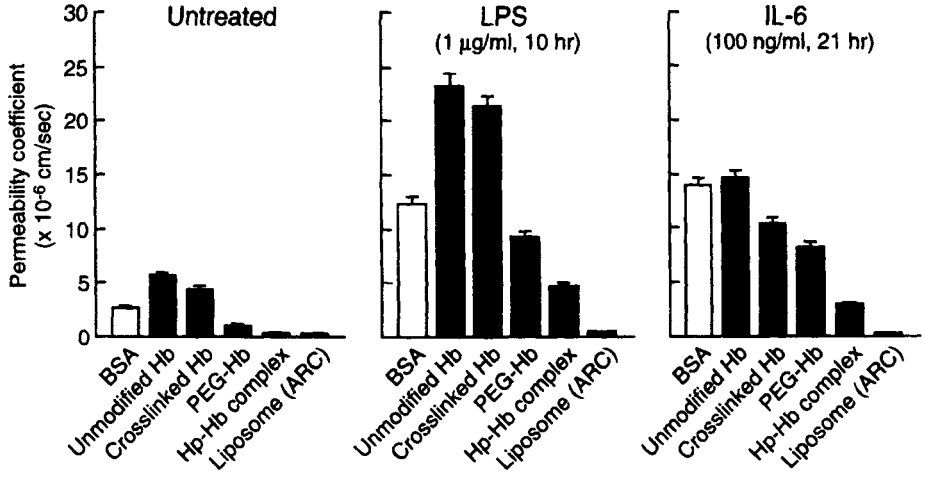

Fig. 7. Permeability characteristics of several Hb preparations in untreated, LPS (1 μg/ml, 10 h)-treated or IL-6 (100 ng/ml, 21 h)-treated monolayers. Means ± SE of 5–10 experiments.

permeabilities. This supports the idea that molecular mass is an essential factor contributing to the permeability characteristics, although we must consider other factors, including molecular shape and net charge [36], to obtain a precise understanding of decreased permeabilities of modified Hbs.

Endothelial barrier functions deteriorate in some pathological situations. In particular, hemorrhagic shock occurring after trauma, which is the most likely situation for the application of HBOCs, is accompanied by decreased endothelial permeability [37,38], in which IL-6 is a major factor regulating the permeability [39]. Oxygen carriers would also likely be infused into trauma patients with concomitant endotoxemia, in which LPS disrupts the endothelial barrier function [40,41]. LPS stimulates monocytes and macrophages to produce several pro-inflammatory cytokines, including IL-6, and these cytokines increase vascular endothelial permeability. Therefore, we examined the transendothelial Hb fluxes with endothelial monolayers pretreated with either IL-6 [39] (100 ng/ml for 21 h) or LPS [40,41] (1 μg/ml for 10 h). The results clearly indicated that acellular Hb derivatives moved more rapidly in settings for the above pathophysiological situations in comparison with untreated monolayers. Interestingly, liposome-Hb showed little endothelial flux even after the pretreatment with IL-6 or LPS. Liposomes having a mean diameter of 200 nm appear to be large enough to prevent extravasation even under pathophysiological conditions.

Discussion

We here provide the first information demonstrating that the extravasation of Hb molecules into the vascular bed is a prerequisite for Hb-induced vasoconstriction, as summarized in Fig. 8. Extravasation could be inhibited by increasing the molecular mass of Hb derivatives, and, therefore, such larger products should have smaller vasoconstrictive activity. Although the exact pathway for extravasation is still unknown, we hypothesize that the endothelial intercellular cleft is a probable route.

Hypertensive reaction due to the vasoconstriction caused by acellular Hb derivatives is one of the unsolved problems in HBOCs [42]. This effect is long-lasting, but the extent of hypertension might be self-limiting. Therefore, this is unlikely to be a serious limitation. Hypertension itself may improve the hypotensive reaction during hemorrhagic shock 43. DCLHb [44] and PEG-Hb [45] will be very effective to eliminate the excessive NO in sepsis. The blood flow in the brain and heart conversely increases during Hb-induced hypertension, while it decreases in other organs [46], presumably because EDHF is the predominant vasodilating factor in the brain and heart (Sakuma I. et al, unpublished observation). The presence of the blood-brain barrier also contributes to prevention of Hb extravasation in the cerebral circulation. Therefore, this differential vasoconstrictive activity can be utilized for the treatment of cerebral ischemic injury [47]. On the other hand, hypertension due to the vasoconstriction of arterioles themselves may impair the oxygen supply to the tissues

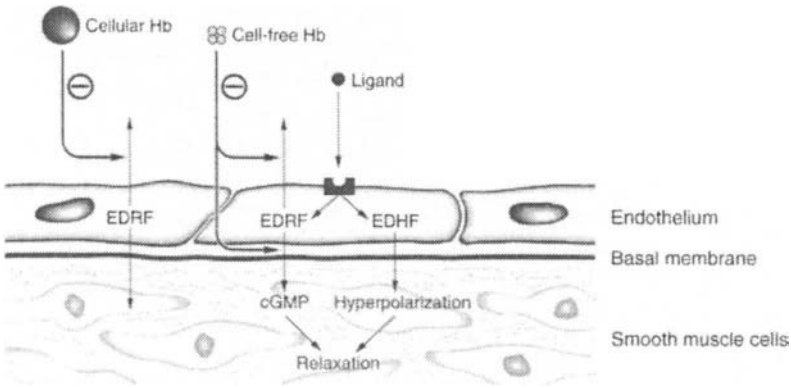

Fig. 8. Possible mechanism of acellular Hb-induced vasoconstriction as a result of abluminal EDRF scavenging.

by reducing the blood flow downstream [48]. It should be again pointed out that the extravasation of Hb might be a prerequisite for the vasoconstrictive reaction. Hb extravasated in turn may induce endothelial damage due to the heme-related oxidative-stress response [49]. The endothelial barrier function should decrease under some pathophysiological conditions; post-ischemic edema in cerebral circulation is closely related to the changes in permeability of the blood-brain barrier [50]. Winslow [42] commented that whether vasoactivity will limit application of acellular HBOCs to clinical use is still open. Thus, the lack of full understanding regarding the consequences of Hb extravasation might be of importance.

Vasoactivity caused by HBOCs does not have a single etiology. Hb may potentiate an adrenergic mechanism [51]. There is some evidence of Hb involvement in the synthesis of endothelin-1 [52,53], although this topic is controversial [54,55]. Hb solution was found to initiate the synthesis of prostaglandins [56] and thromboxanes [57]. It has been emphasized that the extravasation of Hb is likely essential even in these hypothetical mechanisms.

Finally, other Hb-related side effects such as gastrointestinal symptoms and platelet activation may also be related to the removal of NO by Hb. NO plays a significant role in the nonadrenergic noncholinergic nerve-mediated relaxation of gastrointestinal smooth muscle [58]. Endothelium-released NO inhibits platelet function through increasing levels of platelet cGMP. These toxic effects are likely related to the EDRF scavenging by extravasated Hb.

Conclusion

Endothelial permeability of Hb derivatives is a very important factor relating to the difficulties observed with acellular Hb derivatives, especially to Hb-induced vasoconstriction. Endothelial permeability also has a great impact on its circulatory plasma

retention. Thus, safety and efficacy issues are very closel related. Motterlini et al. [49] emphasized the vascular endothelium as the target of acellular Hb derivatives. Successful clinical utilization of HBOCs will require a greater understanding of the interaction between Hbs and endothelium and the consequences of extravasation of Hb in both physiological and pathological situations. We believe that it is possible to engineer acellular HBOCs in order to improve biocompatibility. Our observations also highlight the significance of the cellularity of HBOCs. Great hopes are now held for the development of cellular HBOCs.

Acknowledgments

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 21

Zero-link Polymerization: a New Class of Polymeric Hemoglobins

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Introduction

Hemoglobin solutions have long been proposed as cell-free oxygen carriers to be used as red cell substitutes in infusional fluids. The interest in these compounds stems from their potential clinical applications. They can be sterilized, easily stored, and used in transfusions without blood-group typing. In order to become efficient cell-free oxygen carriers, hemoglobins require at least two modifications: reduction of oxygen affinity and stabilization of the tetrameric structure. Purified hemoglobins, once deprived of the interaction with 2,3-diphosphoglycerate present inside the red cells, have a high oxygen affinity which impairs oxygen release to the tissues. In the rat, untreated hemoglobins are rapidly eliminated in the urine and have a half retention time of only 40–50 min. We understand urine elimination to be the result of the dissociation of tetrameric hemoglobins into dimers. Prevention of dimer dissociation in intramolecularly crosslinked tetramers, prevents glomerular filtration and prolongs the intravascular retention time of the protein [1–4]. Because nephrotoxicity of filterable (dissociable) hemoglobin results, in part, from intratubular precipitation and hemin formation [5,6], these non-filtered tetramers are expected to be less or non-nephrotoxic.

These stable tetramers of hemoglobins are not filtered at the glomerulus however this does not rule out their entry into the renal interstitium where other adverse reactions might occur or result after cellular uptake from the interstitium [1]. Since these stable hemoglobins have a plasma half time in the rat, less than the 6 h for albumin, they have an important degree of extravasation. Post glomerular capillaries are of the fenestrated type with pores having a diameter of 400–600 A and have a high degree of permeability to small solutes and water. Similar to other capillaries they function as if they have large and small pores [8]. Experimental data indicate that they have a high reflection coefficient for albumin. However albumin, does pass into renal lymph ostensibly through the "large pores". The passage of stable tetrameric hemoglobin into posterior lymph was reported by Bleeker et al. [7] suggesting that these materials pass over large pores in other capillaries in the body. We have observed in our preliminary data that these stable tetramers appear also in renal lymph, even in the complete absence of hemoglobinuria.

The passage of a molecule through the "large pores" is affected by the size of the molecule [10]. Sieving curves generated by relating interstitial concentration to renal venous concentration of inulin, myoglobin, horse radish peroxidase and albumin indicate a retarding effect of molecular size [9]. The large pore reflection coefficient of albumin with Mw 64,000 D was found to be 0.24 (maximum reflection is 1.0) while that for γ-globulins with Mw 156,000–160,000 D was 0.44 [10]. Crosslinked tetrameric hemoglobins with a similar molecular weight and diameter to albumin would also be expected to pass across "large pores" and enter into the renal interstitium. Based on available data an increased reflection coefficient would be expected to result as the size of the hemoglobin molecule is increased by polymerization. For example, it would be expected that a polymer of 4 tetrameric hemoglobin units would have a molecular size near 250,000 D and would therefore be further retarded in passage across the renal capillaries. Thus the production of polymerized hemoglobins with a larger molecular diameter (radius) appears to be a rationale goal.

Recently, we have developed in our laboratory several oxygen carriers, including intramolecularly crosslinked tetramers based on human and bovine hemoglobins. Two of these carriers have been extensively characterized and used in physiological trials [1–4]. They are stabilized by a covalent crosslink between the β82 lysines of the opposite subunits in the β-cleft of hemoglobin. The linker is either a fumaryl (4 carbon long) or adipoyl (8 carbon long) residue in bovine hemoglobin and a sebacoyl residue (10 carbon long) in human hemoglobin. The intramolecular crosslink prevents the dissociation of the molecule into dimers and brings the oxygen affinity to a physiologically compatible level. As the molecules do not dissociate into dimers they are not eliminated in the urine and their intravascular retention time is prolonged to about 4 h in the rat and 6 h in the cat [1,4]. These stable tetramers are too small and cannot be used at concentrations higher than 6–8%, because of their colloid oncotic pressure (COP) activity. Thus there is a limitation to their oxygen carrying capacity to about half that of normal blood. Therefore it is very important to develop polymeric hemoglobins which besides producing longer retention times would carry more oxygen per unit of oncotic pressure. The low COP/O_2 capacity ratio would allow the production of infusional fluids with very high oxygen capacity, even higher than in normal blood, yet maintaining COP in the usual physiological range.

Polymerized hemoglobins are commonly obtained by treatment with either glutaraldehyde [11] or raffinose [12]. With both procedures the chemical reaction is random and difficult to control. The obtained polymers are highly heterogeneous, with molecular size distributed over a wide range. The starting material is usually natural, dissociable hemoglobin. This adds to the heterogeneity, because polymerization involves both the tetrameric and dimeric forms of hemoglobin. As reported at meetings, both kinds of polymers have been used in initial clinical trials without any major side effect [13].

Glutaraldehyde is a bifunctional reagent which produces polymers by forming Schiff bases with the amino groups of lysyl residues in adjacent molecules. The Schiff base is not a stable covalent bond. In its reduced form it is more stable. However the

high toxicity of residual glutaraldehyde impurities and of residual non reduced Schiff basis is difficult to control in common scientific laboratories. Polymerization with raffinose and periodate oxydation must be performed in an atmosphere of carbon monoxide, very difficult to remove completely in non industrial settings.

In the attempt to reduce heterogeneity we adopted the strategy of polymerizing hemoglobins intramolecularly crosslinked, so as to avoid the presence of dimeric components. Also we decided to use chemical reactions, which do not leave potentially toxic chemical linkers in the polymers. Our data have been obtained using two intramolecularly crosslinked hemoglobins: sebacoyl crosslinked human hemoglobin (indicated as sebacoyl-HbA or DecHbA), and adipoyl crosslinked bovine hemoglobin (indicated as adipoyl-HbBv). The main characteristics of sebacoyl-HbA are as follows. The p_{50} is near 34 mmHg at 37°C, with the oxygen binding cooperativity Hill's parameter $n = 2.2$. In comparison, whole blood has p_{50} near 27 mmHg and $n = 2.8$. The half-time of intravascular retention is near 4.0 h in the rat and 6.5 h in the cat. Spectrophotometric analysis of arterial and venous plasma of infused rats reveal that in venous blood at least 50% of its oxygen content is released to tissues [14]. Similar characteristics are present in adipoyl-HbBv, which has a very low oxygen affinity with a p_{50} near 50 mmHg and a value of n =near 1.9 (unpublished).

Experimental procedures

The zero-link polymerization reaction

The chemistry of the reaction is schematically shown in Fig. 1. The water soluble carbodiimide EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide] activates the side chain carboxylate groups of the hemoglobin surface, forming a highly reactive

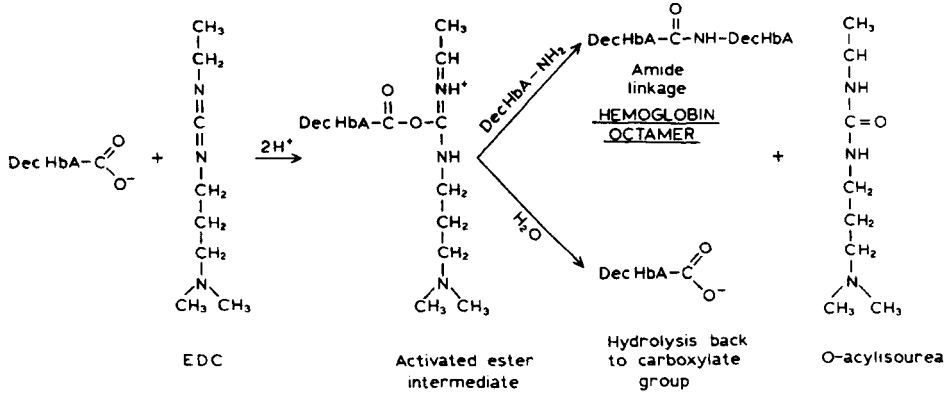

Fig. 1. Schematic representation of the zero-link polymerization with EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide).

and short lived *O*-acylisourea derivative. This complex is formed from C-terminal and Glu or Asp side chains carboxylic group. This activated species react with the side chains of the lysyl residues (primary amines) of another hemoglobin molecule to form a stable amide bond, similar to a peptide bond. The activated species can also react with sulfhydryl groups to generate thiol ester linkages and with hydroxyl group forming ester bonds (Fig. 2).

The isourea by-product formed during the reaction is soluble in aqueous solution and can be easily removed by dialysis. Thus, hemoglobin molecules are polymerized directly by covalent either CO—NH or ester bonds between groups of adjacent tetramers in the absence of chemical linkers. This reaction can be defined as "zero-link" [15]. Although the EDC coupling reaction is quite efficient, the activated carboxyl group may be hydrolyzed by water molecules rather than react with the amino or other groups. Staros et al [16] have developed a two-step approach to enhance the yield of amide bond formation. In this procedure, the addition of *N*-hydroxysulfosuccinimide (sulfo-NHS) to the carbodiimide reaction results in the formation of an intermediate sulfo-NHS ester, which in turn reacts with the amino, thiol and hydroksyl groups. This reaction is schematically represented in Fig. 3.

The increased stability of the sulfo-NHS ester of the hemoglobin carboxyl groups, compared with that of their corresponding *O*-acylisourea intermediates, decreases the loss of the activated carboxyl through hydrolysis. Thus the relative efficiency of the EDC-activated carboxyl group channeled through the nucleophilic attack pathway is increased in the presence of sulfo-NHS. This implies the possibility of modulating the extent of polymerization by changing the relative amounts of sulfo-NHS and EDC in the reaction mixture.

Zero-link polymerization of sebacoyl-HbA in aerobic and anaerobic condition (PolyA)

Oxy-sebacoyl-HbA (concentration 6%) was polymerized in the presence of 5 mg/ml EDC and 1 mg/ml sulfo-NHS in 0.1 M MOPS pH = 6.7 at room temperature for 3 h. The reaction was stopped by addition of glycylglycine to a final concentration of 0.1 M, followed by dialysis against 0.1 M glycine at pH = 8. The solution was

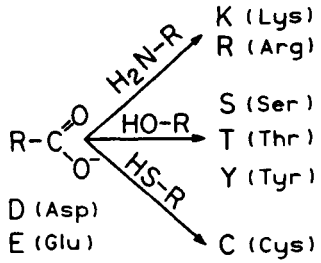

Fig. 2. Amino acid side chains potentially reacting with EDC activated carboxyl groups on the surface of hemoglobin.

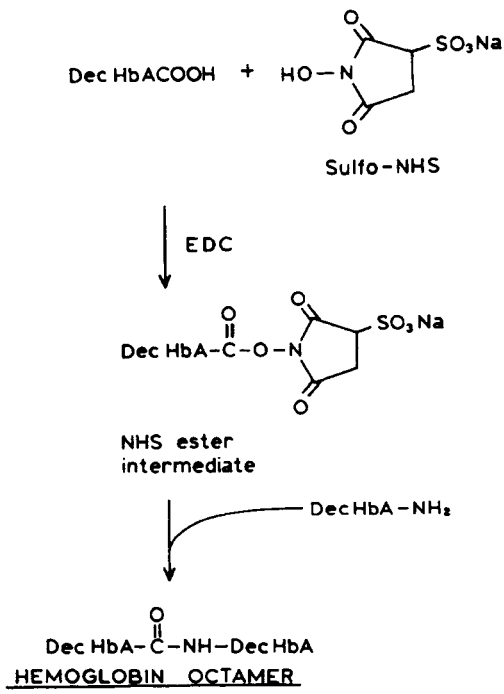

Fig. 3. Two step reaction of hemoglobin molecules with EDC in the presence of sulfo-NHS.

dialyzed against 0.015 M Tris/acetate pH = 8.2. The mixture of polymers was purified by preparative Waters HPLC using a gradient elution of 0.015 M Tris/acetate pH = 8.2 to 0.015 M Tris/acetate, 0.5 M sodium acetate pH = 7.4. The elution profile of the chromatography is shown in Fig. 4(A). The marked fractions were collected and tested for p_{50} and the average radius. Oxygen binding isotherms

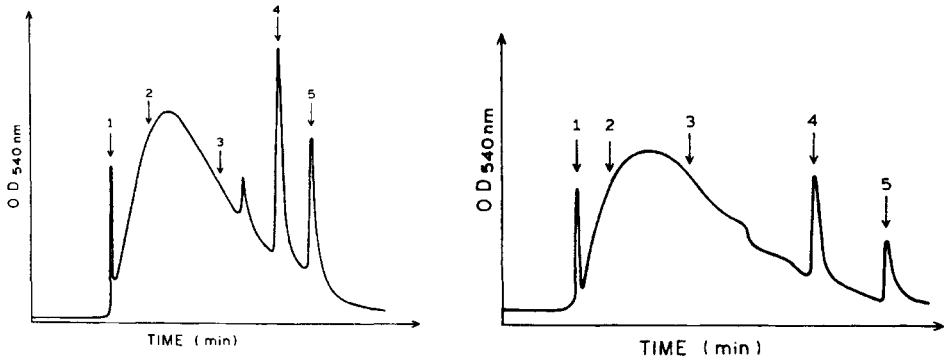

Fig. 4. Anion-exchange chromatography on DEAE resin of zero-link polymerized deoxy-sebacoyl-HbA (A) and zero-link polymerized oxy-sebacoyl-HbA (B).

were measured using a Hemoxanalyzer apparatus (TCS Medical Products Company, Huntingdon Valley, PA) for dilute solutions of hemoglobin in 0.1 M Tris-Cl pH = 7.4 at 37°C. The average molecular radius of the molecules in solution was estimated using dynamic light scattering (DynaPro-801 Molecular Size Detector, Protein Solutions Inc., Charlottesville, VA). It should be stressed that the scattering data are biased in favor of the largest molecules in solution. All data are reported in Table 1.

Deoxy-sebacoyl-HbA was polymerized with EDC identically, but in anaerobic conditions. Purification of the polymers mixture was done under the same conditions as for oxy-sebacoyl-HbA. The elution profile is presented in Fig. 4(B). The p_{50} and average molecular radius of the marked fractions are reported in Table 1.

All the obtained polymers were readily soluble in water. The data in Fig. 4 clearly indicate the presence of several distinct populations of polymers. In both kinds of polymers, fractions 1 probably still contain non-polymerized sebacoyl-HbA, fractions 2 and 3 suggest the presence of polymers centered at 3-mer and 8-mer of sebacoyl-HbA respectively. Fractions 4 and 5 include highly polymerized species. The sharp chromatographic resolution of the highly polymerized fractions and of the non-polymerized ones underscore the usefulness of ionic exchange chromatography for reducing the heterogeneity of polymerized sebacoyl-HbA.

Table 1

Data on sebacoyl-HbA reacted with EDC in the presence of sulfo-NHS in anaerobic and aerobic conditions. Average molecular weights are estimated from the respective radii, assuming that the polymers still have a spherical shape. Normal Mw = $(\text{radius}/3.2)^3 \times 64,000$. 3.2 nm is the radius of sebacoyl-HbA, as expected for tetrameric hemoglobin with Mw = 64,000 D. The fraction numbers are those shown in the respective chromatographies (Fig. 4)

	Sample	p_{50} (mmHg)	Average radius (nm)	Normal molecular weight (kD)
	sebacoyl HbA	34	3.2	64
D	crude polymers	16.7	6.1	443
E	fraction 1	20.5	3.8	107
O	fraction 2	20.5	4.5	178
X	fraction 3	19.5	6.6	561
Y	fraction 4	18.3	9	1423
	fraction 5	12.9	10.2	2079
	crude polymers	11.7	5.5	324
O	fraction 1	12.4	3.7	99
X	fraction 2	9.8	4.2	144
Y	fraction 3	10.9	5.2	274
	fraction 4	11.3	7.6	857
	fraction 5	11.4	10.5	2260

Zero-link polymerization of adipoyl-HbBv (PolyB)

Oxy-adipoyl-HbBv (concentration 6%) was polymerized in the presence of 5 mg/ml EDC and 1 mg/ml sulfo-NHS in 0.1 M MOPS pH = 6.7 at 37°C for 90 min. The reaction was stopped by addition of glycylglycine to a final concentration of 0.1 M, followed by dialysis against 0.1 M glycine at pH = 8. The solution was dialyzed against 0.015 M Tris/acetate pH = 8.2. The mixture of polymers was purified by preparative Waters HPLC using a gradient elution of 0.015 M Tris/acetate pH = 8.2 to 0.015 M Tris/acetate, 0.5 M sodium acetate pH = 7.4. The elution profile of the chromatography is shown in Fig. 5(A). The p_{50} and the average radius were measured for marked fractions. These data are presented in Table 2.

Deoxy-adipoyl-HbBv was polymerized in the same way, only under anaerobic conditions. The chromatographic profile is shown in Fig. 5(B). P_{50} and molecular radius of collected fractions are reported in Table 2.

Also in this case ionic exchange chromatography very efficiently recognized the molecular size of the polymers. Table 2 clearly shows that adipoyl-HbBv polymerizes more readily than sebacoyl-HbA, especially in its deoxy-form. It also produces polymers with physiologically competent oxygen affinity even for highly polymerized material. The low affinity of the polymers obtained under anaerobic conditions is unprecedented. During the manipulations of these chromatographic fractions, even those with highly polymerized material like fractions 4 and 5, the viscosity of the solutions did not appear to be higher than that of normal unpolymerized hemoglobin.

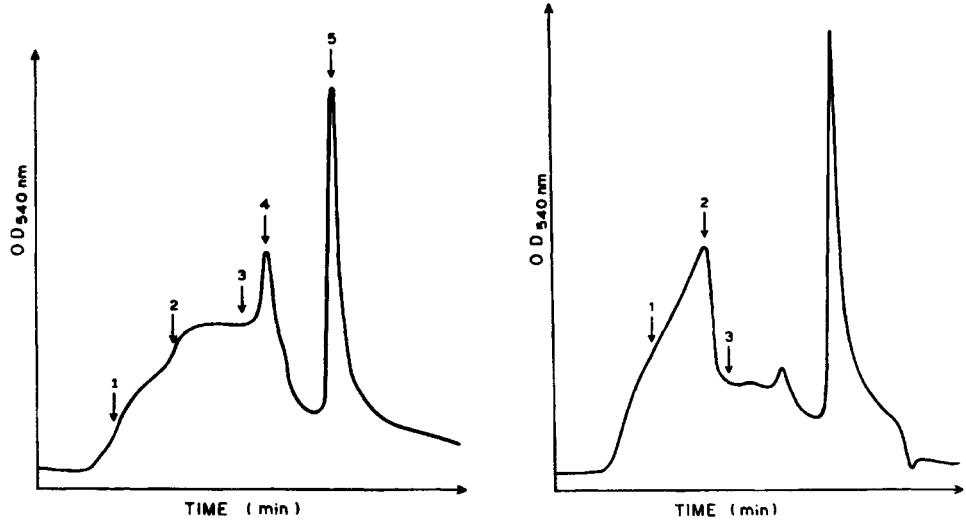

Fig. 5. Anion-exchange chromatography on DEAE resin of zero-link polymerized deoxy-adipoyl-HbBv (A) and zero-link polymerized oxy-adipoyl-HbBv (B).

Table 2

Data on adipoyl-HbBv reacted with EDC in the presence of sulfo-NHS in anaerobic and aerobic conditions. Average molecular weights are estimated from the respective radii, assuming that the polymers still have a spheral shape normal Mw = $(\text{radius}/3.2)^3 \times 64{,}000$. The radius of adipoyl-HbBv is 3.2 nm, as expected for tetrameric hemoglobin with Mw = 64,000 D. The fraction numbers are those shown in the respective chromatgraphies (Fig. 5).

	Sample	p_{50} (mmHg)	Average radius (nm)	Normal molecular weight (kD)
	sebacoyl HbBv	53.1	3.2	64
D	crude polymers	33.2	14.4	5832
E	fraction 1	51.9	4.7	203
O	fraction 2	46.5	5.2	274
X	fraction 3	42.3	9.1	1471
Y	fraction 4	N/A	13.4	4699
	fraction 5	N/A	30.6	55,962
O	crude polymers	19.5	15.5	7273
X	fraction 1	19.4	6.1	443
Y	fraction 2	20.1	7.0	669
	fraction 3	20.1	9.5	1674

Tryptic maps of entire HbA and its zero-link polymerized form PolyA

Due to impossibility to separate the α and β subunits of polymeric hemoglobin, the tryptic peptide maps of PolyA were compared to those obtained from the entire hemoglobin molecule. Before tryptic cleavage, 50 nmol samples of total protein from HbA either before or after polymerization treatment were *S*-pyridylethylated by dissolving in 0.25 MTris-HCl, 1 mM EDTA, 6 M urea and 16 µl of 10% aqueous solution of β-mercaptoethanol. After stirring 1.5 h under nitrogen 5 µl of 4-vinylpyridine was added and the reaction took place for 1.5 h at room temperature. The samples were desalted by reverse-phase HPLC using a 4.6 × 250 mm Vydac 214TP54 C_4 column. The column was equilibrated with 0.1% aqueous trifluoroacetic acid (TFA) as solvent A. The pyridylethylated protein was eluted using a gradient with 90% acetonitrile in solvent A (solvent B). The gradient was 0–100% B in 30 min.

The pyridylethylated protein was dried in freeze-dried and redissolved in 1 ml of 0.1 M ammonium bicarbonate. Tryptic digestion was performed overnight at room temperature using 1 : 50 weight ratio of trypsin to the protein. The resulting solution was then directly injected onto a 4.6 × 250 mm Vydac 218TP54 C18 reverse-phase column. The column was equilibrated with solvent A and peptides were eluated with a linear gradient from 0% to 50% over 60 min of solvent B. Peptides were monitored at 280 nm so as to detect only the peptides containing aromatic residues. The elution profile is shown in Fig. 6. The indicated fractions were collected and used for amino acid sequencing. Sequences were determined using a Heweltt-Packard protein sequencer G-1005A. The peptide compositions of the collected fractions is presented in Table 3. The tryptic fragments of human hemoglobin are shown in Table 4.

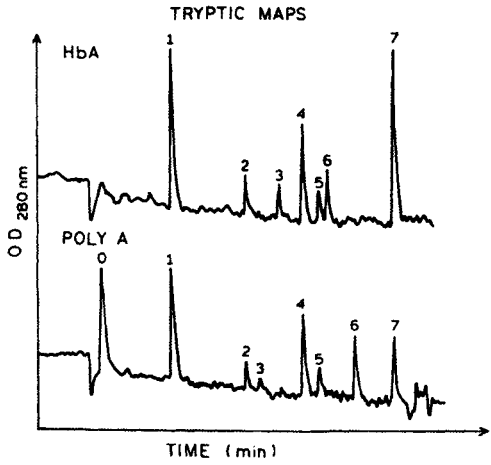

Fig. 6. Tryptic maps of entire HbA (Top) and of its zero-link polymerized form PolyA (bottom) with average radius $R = 6.6$ nm. The chromatography on C18 columns was monitored at 280 nm, therefore only peptides containing aromatic residues were detected. Note the different position of peptides 3 and 6, respectively.

Table 3

Composition of tryptic peptides included in the peaks revealed by monitoring at 280 nm the chromatography of the tryptic digest of entire HbA molecule and of the zero-link polymers of sebacoyl-HbA (PolyA). The peak's numbers are those indicated in the respective peptide maps. "Non-specific" means a cleavage non specific for trypthin producing a portion of the peptide shown in parenthesis. The fraction numbers are those indicated in the elution profile in Fig. 6.

	Natural HbA		Polymers of sebacoyl-HbA	
Peak No.	Tryptic peptides included	Amount of each (pM)	Tryptic peptides included	Amount of each (pM)
0			non-specific (4α)	200
1	3α	100	3α	100
	1α	50	1α	10
2	4α	300	4α	400
3	13β	100	13β	300
			4α	150
4	2β	200	2β	20
			non-specific (6α)	
5	5α	400	5α	300
	6α	200	6α	150
6	5α	800	9α	100
	6α	200	5β	100
			2β	100
7	4β	200	4β	200

Table 4

Typical fragments of human hemoglobin

α-Chain		
Fragment	Residues	Sequence
01	1–7	VLSPADK
02	8–11	TNVK
03	12–16	AAWGK
04	17–31	*VGAHAGEYGAEALER*
05	32–40	MFLSFPTTK
06	41–56	TYFPHFDLSHGSAQVK
07	57–60	GHGK
08	61	K
09	**62–90**	**VADALTNAVAHVDDMPNALSALSDLHAHK**
10	91–92	LR
11	93–99	VDPVNFK
12	100–127	LLSHCLLVTLAAHLPAEFTPAVHASLDK
13	128–139	FLASVSTVLTSK
14	140–141	YR
β-Chain		
Fragment	Residues	Sequence
01	1–8	VHLTPEEK
02	9–17	SAVTALWGK
03	18–30	VNVDEVGGEALGR
04	**31–40**	**LLVVYPWTQR**
05	**41–59**	**FFESFGDLSTPDAVMGNPK**
06	60–61	VK
07	62–65	AHGK
08	66	K
09	67–82	VLGAFSDGLAHLDNLK
10	83–95	GTFATLSELHCDK
11	96–104	LHVDPENFR
12	105–120	LLGNVLVCVLAHHFGK
13	121–132	*EFTPPVQAAYQK*
14	133–144	VVAGVANALAHK
15	145–146	YH

The peptides involved in potential intermolecular crosslinks are bold.

Viscosity of the polymers

Relative viscosity was measured at 37°C using an Ostwald viscometer. The polymers were dissolved in lactated Ringer. The data in Table 5 indicate that at the concentrations generally used for animal trials the viscosity of the polymers is similar to that of plasma.

Table 5

Relative vicosity at 37°C of PolyA and PolyB in lactated Ringer solutions. PolyA was obtained in anaerobic condition with $R = 6.6$ nm and PolyB was obtained in anaerobic condition with $R = 12$ nm.

Polymer	Concentration (mg/ml)	Relative viscosity
PolyA	12.0	2.2405
PolyA	8.0	1.6459
PolyA	7.0	1.5156
PolyA	6.0	1.4202
PolyB	7.0	1.5119
plasma	N/A	2.0148

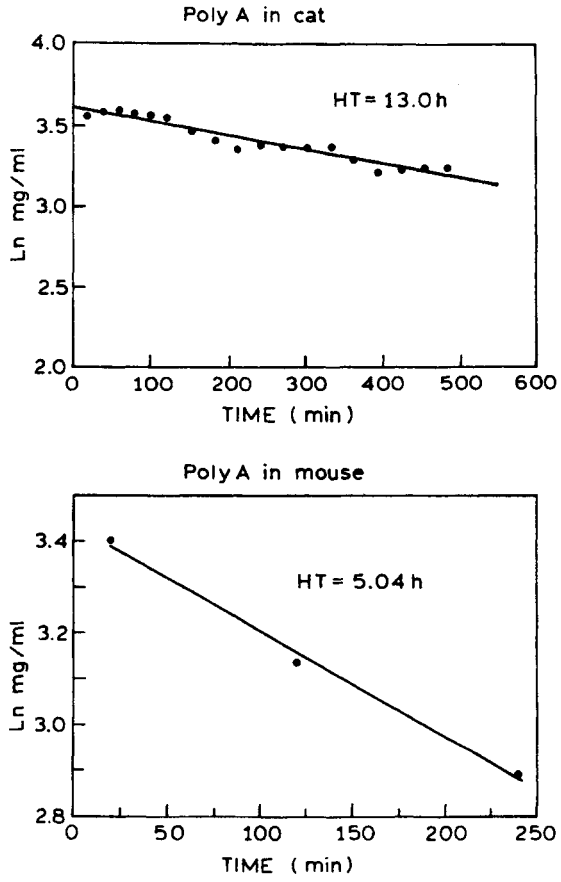

Fig. 7. Half time of retention of PolyA (average radius $R = 6.6$ nm) in the cat (top) and in the mouse (bottom) after 30% exchange transfusion. PolyA concentration was 6%.

Oncotic activity

The oncotic activity of the polymers was measured with a WESCOR 4010 oncometer (Wescor Inc., Logan, UT). Only isooncotic solutions were used for physiological trials. The solutions were in lactated Ringer and tested against lactated Ringer. The data are presented in Fig. 8. As expected the polymerized hemoglobins have lower COP/g than tetramer hemoglobins.

Intravascular retention time

Experiments were performed on the cat, the rat and the mouse. Measurements in the cat and mouse were performed in the laboratory of Dr R. Koehler (Johns Hopkins University), while experiments on the rat were done in the laboratory of Dr Barbara Matheson (Department of Physiology, University of Maryland, Dental School). The femoral vein of these animals were cannulated for the infusion of the polymers in lactated Ringer solutions. The animals were anesthetized and left breathing room air spontaneously. Exchange transfusions were performed to 30–50% substitution of circulating blood [3,4]. Only PolyA and PolyB obtained by polymerization under anaerobic conditions were used for the infusions. The average radii of the two polymers were near 6 and 12 nm respectively. In the rat the half time of retention was 11 ± 1 h, in the cat it was 13 ± 1 h. In the mouse the half time of retention was close to 5 h. This was a welcome result that proved the viability of these polymers also in very small animals, whose intravascular retention time is notoriously very short. As usual with hemoglobin derivatives, the blood pressure in the rat and in the cat (N/A for the mouse) showed a 10–20% transient increase after infusion. No indication was noticed

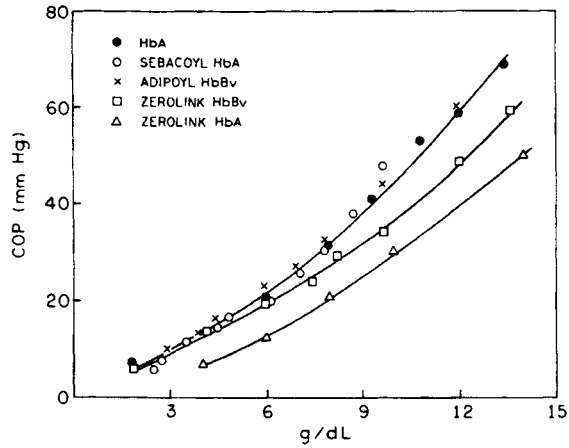

Fig. 8. Colloid osmotic pressure of HbA, sebacoyl-HbA, adipoyl-HbBv, PolyA obtained in anaerobic condition with $R = 6.6$ nm and PolyB obtained in anaerobic condition with $R = 12$ nm.

of either kidneys or heart discomfort. There was no urine elimination in any of the treated animals. Recently repetitive exchange transfusions on awake cats at 10–12 h distance were well tolerated and the animals appeared to be normal after two-three days, when they were sacrificed for additional experiments. In one case the cat was not sacrificed and became the pet of a collaborator.

Discussion

The data in Tables 1 and 2 demonstrate that crosslinked tetramers of human and bovine hemoglobins can be polymerized by intermolecular crosslinking based on the activation of carboxyl groups on the surface of the tetramers. The active carboxyl groups form covalent bonds with several groups present on the surface of an adjacent molecule, forming a so-called "zero-link". Extensive polymerization can be obtained with both human and bovine hemoglobins. Polymerization involves mainly lysyl residues on the surface of hemoglobin. Therefore the extent of polymerization can be modulated by addressing the chemical reactions to the lysyl residues with the highest chemical reactivity. The nucleophilic attack of lysyl residues (responsible for both kinds of polymerization reactions) is produced by deprotonanted lysines. The pK of the lysyl residues is near 9.0 or above, however, electrostatic and steric conformations may lower that pK on the surface of hemoglobin. For example the lysines at $\beta 59$ and $\beta 61$ are near other positively charged groups, therefore their pK is probably lower than that of the other lysines. Decreasing the pH of the chemical treatment toward neutrality will favor the reaction of low-pK lysines. Therefore we decided to choose pH = 6.7 as the best condition for the reaction.

We wanted to avoid excessive viscosity of the solution which is directly proportional to the size of the polymers. Preliminary data suggest that the oxygen affinity and viscosity increase with the size of the polymers. Therefore, at present, we have a preference for the smaller hemoglobin polymers, which appear to be the best compromise among size distribution, oxygen affinity, viscosity and COP.

As shown in Figs. 4 and 5 anion exchange chromatography gave the possibility of obtaining fractions with different molecular size. The data presented in the Tables 1 and 2 show that the oxygen affinity of the resulting polymers was directly proportional to the extent of polymerization. Also, adipoyl-HbBv produced polymers with lower oxygen affinity. Thus, polymers can be obtained with p_{50} near 20 mmHg using human hemoglobin reacted in anaerobic condition or p_{50} near 30 mmHg or even higher using bovine hemoglobin.

Also, from the data reported on peptide maps (Fig. 6, Tables 3 and 4) it appears that, at least for PolyA, polymerization is not entirely random. Peptides 3 and 6 (Fig. 6) had a different position in the polymers and revealed the presence of peptides both from the α and β subunits only after polymerization, suggesting the presence of regions of preferential interaction. Table 3 suggests that the peptide 4α is linked to peptide 13β (although the stoichiometry of 1:2 is not clear at present). Also peptide 9β

would be linked to peptides 4β and 5β (also in this case there is a 1:2 stoichiometry which must be clarified). It may be stressed that lysine β59 and β61, mentioned above as low pK lysines, are included in 5β and 6β (the last non detectable in the tryptic peptides maps).

The oncotic activity per unit weight of these compounds in lactated Ringer's solution was lower than that of either unmodified or intramolecularly crosslinked hemoglobins. This was expected, because of their much larger molecular weight. As shown in Fig. 8 the oncotic activity of all of these compounds showed a strong dependence on protein concentration. The various preparations are isooncotic at 8–9 g/dl. They become rapidly hyperoncotic at concentration above 10%. PolyA showed COP near physiological values at 12% concentration, thereby approaching the oxygen carrying capacity of blood.

Acknowledgments

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Abbreviations

Adipoly-HbBv	bovine hemoglobin crosslinked with an adipoyl residue (6 carbons)
Sebacoyl-HbA or DecHbA	human hemoglobin crosslinked with a sebacoyl residue (10 carbons)
PolyA	polymerized sebacoyl-HbA
PolyB	polymerized adipoyl-HbBv
COP	colloid oncotic pressure
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
sulfo-NHS	*N*-hydroxysulfosuccinimide
MOPS	3-(*N*-morpholino) propane-sulfonic acid
EDTA	(ethylenedinitrilo)tetraacetic acid, disodium salt

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 22

Recombinant Hemoglobins with Low Oxygen Affinity and High Cooperativity

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Introduction

The need for a safe, reliable blood substitute has long been recognized (for recent reviews, see refs [1,2]). Hemoglobin (Hb)-based oxygen carriers are a potential source of blood substitutes during emergency medical situations. In order to make these oxygen carriers practical, commercially viable blood substitutes, we need to design the Hb molecule to fulfill the following three essential requirements: (1) The designed Hbs should deliver oxygen efficiently, i.e. they should combine with and unload oxygen cooperatively like human normal adult hemoglobin (HbA) inside red blood cells. (2) In the extra-cellular environment, because of the absence of allosteric effectors, such as 2,3-diphosphoglycerate (2,3-DPG), which serve to lower the oxygen affinity of Hb within the red blood cell, it is essential to have Hbs with low oxygen affinity. (3) The designed Hbs should be stable against the oxidation of the heme iron atom from the ferrous to the ferric state and should not dissociate readily into the dimers of Hb. Several naturally occurring mutant Hbs, such as Hb Kansas (β102Asn $\rightarrow$ Thr) [3] and Hb Titusville (α94Asp $\rightarrow$ Asn) [4], have decreased cooperativity and a strong tendency to dissociate into dimers upon oxygenation. Thus, this type of Hb is unsuitable as an acellular oxygen carrier. With the development in our laboratory of an expression system to produce authentic human normal adult hemoglobin (HbA) in *Escherichia coli* [5], we are in a position to design and express any recombinant Hbs needed for our research. Some of the recent biochemical-biophysical studies on rHbs designed in our laboratory are described in our publications [6–12]. In this chapter, we shall concentrate on the first two requirements (i.e. high cooperativity and low oxygen affinity) for the design of Hbs as potential oxygen carriers.

The working hypothesis for our design of low oxygen affinity Hbs with high cooperativity is that if we can stabilize or provide extra stability to the deoxy (or T) quaternary structure of the Hb molecule, we should have Hbs with low oxygen affinity. The starting point for our design of low oxygen affinity Hbs with high

cooperativity comes from the information derived from the crystal structures of HbA in both deoxy- and oxy-forms [13–17] as well as the structural and functional properties of known human abnormal Hbs [17–19]. It is known that the Hb molecule has a lower oxygen affinity in the T state than in the oxy (or R) quaternary structure. Based on a comparison of the detailed structural features of HbA in deoxy- and oxy-forms, Perutz and colleagues [13–16] have shown that during the transition from the deoxy to the oxy state, the $\alpha_1\beta_2$ subunit interface undergoes a sliding movement (Fig. 1), while the $\alpha_1\beta_1$ subunit interface remains nearly unchanged. Both subunit interfaces are characterized by specific H-bonds, salt bridges, and non-covalent interactions. It has also been found that human Hbs with mutations in the $\alpha_1\beta_2$ subunit interface exhibit altered oxygen affinity and cooperativity [17–19].

For our present purpose, there are three types of low oxygen affinity mutants of HbA. The first type of mutants, which includes Hb Kansas (β102Asn → Thr) [3] and Hb Titusville (α94Asp → Asn) [4], exhibits greatly reduced cooperativity, and dissociates readily into dimers upon oxygenation. In the crystal structure of oxyHbA, the $\alpha_1\beta_2$ interface includes a characteristic H-bond between α94Asp and β102Asn, which is broken in the deoxy-state [14–16]. It appears that a mutation at either α94 or β102 can greatly destabilize the oxy (or R)-form of this type of mutant Hb. Due to their low cooperativity and strong tendency to dissociate into dimers, this type of low affinity hemoglobin is not suitable as an oxygen carrier. The second type

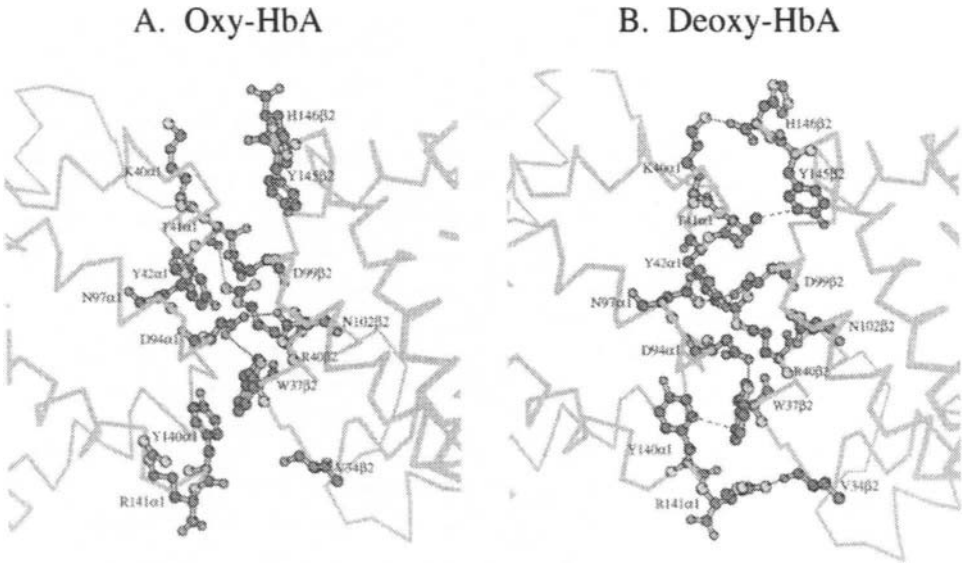

Fig. 1. Structural features of the $\alpha_1\beta_2$ subunit interface for oxy-HbA and deoxy-HbA showing some inter-subunit H-bonds and contacts. The structures of oxy- and deoxy-HbA were obtained from the Brookhaven Protein Data Bank, entries 1HHO and 3HHB, respectively. This figure was prepared using the Molscript software package [38].

of low oxygen affinity Hb mutants, such as Hb Yoshizuka (β108Asn → Asp) [20,21] and Hb Presbyterian (β108Asn → Lys) [21–25], exhibits good cooperativity, and does not appear to dissociate into dimers as readily as do Hb Kansas and Hb Titusville. According to the published crystal structures of oxy- and deoxy-HbA, β108Asn forms an H-bond with α103His in the $\alpha_1\beta_1$ subunit interface [14,17]. However, a recently determined, highly refined structure of deoxy-HbA shows that α103His does not form an H-bond with β108Asn, but instead it forms an H-bond with β131Gln (J. Tame, unpublished results; personal communication). Bonaventura et al. [26] proposed that the repulsion between excess positive charges introduced by mutations of the amino acid residues bordering on the central cavity can lead to the widening of the cavity, thus lowering the oxygen affinity of Hb. O'Donnell et al. [21] have discussed the issue regarding the mechanism of the low oxygen affinity of these two mutant Hbs, in light of their own results on these two mutant Hbs and the suggestion of Bonaventura et al. [26]. It is interesting to note that both Hb Presbyterian and Hb Yoshizuka exhibit low affinity and high cooperativity, but the amino acid substitutions of these mutants have opposite charge, i.e. Lys vs Asp. More research is needed to understand the molecular basis which gives rise to the low oxygen affinity and high cooperativity in this class of mutant Hbs. The third type of low oxygen affinity Hb mutants has amino acid substitutions in the $\alpha_1\beta_2$ subunit interface, such as rHb Mequon (β41Phe → Tyr) [27] and rHb (α96Val → Trp) [28]. These two rHbs exhibit high cooperativity, are also quite stable against oxidation, and do not dissociate into dimers. Baudin et al. [27] hypothesized that in rHb Mequon, the β41Tyr in the β_2-chain can participate in new electrostatic interactions in the $\alpha_1\beta_2$ subunit interface with amino acid residues of the α_1-chain, thus stabilizing the deoxy (T)-quaternary structure so as to give rise to low oxygen affinity.

Our strategy for designing low oxygen affinity Hbs is to stabilize the deoxy (T)-quaternary structure and not perturb the oxy (R)-quaternary structure. Our first rHb with low oxygen affinity and high cooperativity has an amino acid substitution at the $\alpha_1\beta_2$ subunit interface, namely, α96Val → Trp. This rHb (α96Val → Trp) exhibits about the same oxygen affinity and cooperativity as those of HbA plus 2,3-DPG [28], a necessary requirement if this type of Hb is to function as an oxygen carrier in the extra-cellular environment outside red blood cells (i.e., in the absence of the physiological allosteric effector, 2,3-DPG, found in red blood cells). This rHb can switch from the R-quaternary structure (i.e., in the CO form) to the T-quaternary structure without changing the ligation state when the temperature is lowered and/or when an allosteric effector, inositol hexaphosphate (IHP), is added. This indicates that the T-quaternary structure of this rHb is stabilized. Our next step is to look into the effect of the mutation at β108Asn in combination with the mutation at α96Val on the structure-function relationship in these rHbs. In this chapter, we describe briefly some of our recent biochemical-biophysical studies on Hbs with the desirable properties of low oxygen affinity and high cooperativity.

Materials and methods

Plasmids, strains, chemicals, and restriction enzymes

The construction and expression of our hemoglobin plasmid, pHE2, have been described in our previous publications [5,29]. The mutation of α96Val → Trp and the construction of plasmid pHE202 for the expression of rHb (α96Val → Trp) were reported previously [28]. The mutation of β108Asn → Lys was made in a similar manner, except that the synthetic oligonucleotide used was 5′-CAAACTAGAAC-CTTACCCAGCAG-3′, to produce the plasmid, pHE240, for the expression of rHb Presbyterian (β108Asn → Lys). The plasmid pHE249 for the expression of the double mutant, rHb (α96Val → Trp, β108Asn → Lys, was constructed by ligation of the 0.76-kb BamH I–BstB I fragment of pHE202 and the 6.1-kb BamH I–BstB I fragment of pHE240.

The plasmids for expression of rHbs were transformed into *E. coli* JM109, *E. coli* BL(DE3)/pMS421, or *E. coli* BL(DE3-DR)/pMS421. *E. coli* JM109 was purchased from Promega. *E. coli* BL21(DE3) was provided by Dr Maureen Gilmore-Herbert of Yale University. The plasmid pMS421, which contains I_q and was provided by Dr William R. McClure of Carnegie Mellon University, was transformed into *E. coli* BL21(DE3) to give *E. coli* BL(DE3)/pMS421. *E. coli* BL(DE3-DR)/pMS421 was derived in our laboratory from strain BL21(DE3), and is resistant to common phages.

Chemicals and restriction enzymes were purchased from major suppliers, such as Fisher, Sigma, Bio-Rad, Boehringer Mannheim, New England BioLabs, Pharmacia, Promega, United States Biochemicals, Inc., and were used without further purification.

Growth of cells

E. coli cells were grown in a 10-l Microferm fermentor (New Brunswick Scientific, Model BioFlo 3000) at 30°C until the optical density at 600 nm reached 10. Expression of rHb was induced by adding isopropyl β-thiogalactopyranoside (Sigma) to 0.1–0.4 mM. The culture was then supplemented with hemin (20–50 mg/l) and glucose (10–20 g/l), and the growth was continued for at least 4 h. The cells were harvested by centrifugation and stored frozen at −80°C until needed for purification. For details, refer to [5,29].

Isolation and purification of recombinant hemoglobins

The detailed procedures for the isolation and purification of rHbs are described in our previous publications [5,8,28,29]. After the Q-Sepharose fast-flow column step, the rHb samples were oxidized and reduced in order to convert the abnormal heme conformation in the β-chains of the rHbs to the correct one. The converted rHb samples were then purified through the Mono S chromatographic step.

Characterization of recombinant hemoglobins

For the amino-terminal analysis, automated cycles of Edman degradation were performed with an Applied Biosystems gas/liquid-phase sequencer (Model 470/900A) equipped with an on-line phenylthiohydantoin amino acid analyzer (Model 120A). The electrospray ionization mass spectrometric analyses of rHbs were performed on a VG Quattro-Bio-Q mass spectrometer (Fisons Instruments, VG Biotech, Altrincham, U.K.). For details on both amino-terminal and mass spectrometric analyses of our rHbs, refer to Shen et al. [5,29].

Equilibrium oxygen-binding properties of recombinant hemoglobins

The oxygen dissociation curves of rHbs were measured by a Hemox Analyzer (TCS Medical Products, Huntington Valley, PA) as a function of temperature (from 10 to 37°C) and pH (from 6.2 to 8.6) in 0.1 M sodium phosphate. The concentration of Hb used for these measurements was about 0.1 mM (in terms of heme). Partial O_2 pressure at 50% saturation (p_{50}) and the Hill coefficient (n_{max}) were determined from each oxygen dissociation curve. The accuracy of our p_{50} measurements was ±5% or better and that of n_{max} was ±7% or better. For details, refer to refs [5,28,29].

Carbon monoxide binding kinetics of recombinant hemoglobins

The kinetics of the binding of CO to rHbs were investigated using an OLIS stopped-flow apparatus (OLIS, Bogart, GA) (with a dead time of 3 msec) at 20°C. In order to maintain anaerobic conditions in the stopped-flow apparatus, a 10-ml solution of degassed 0.1 M sodium phosphate buffer at pH 8.5 containing 50 mg dithionite was loaded into the stopped-flow system the day before the kinetic measurements. The water bath in the stopped-flow apparatus was bubbled with Ar gas overnight and during the experiment.

One of the syringes in the stopped-flow apparatus contained a deoxy-rHb sample in 0.1 M sodium phosphate at pH 7.0 and the other syringe contained CO-saturated 0.1 M sodium phosphate at pH 7.0 (the concentration of CO is 1 mM). The CO association kinetic experiments were monitored at 540 and 420 nm and the typical time window was 0.1 s. For experiments at 540 nm, the final concentration of rHb was 50 μM and for those at 420 nm, the final rHb concentration was 10 μM.

^{1}H-NMR investigations of recombinant hemoglobins

^{1}H-NMR spectra of rHbs were obtained from a Bruker AM-300 NMR spectrometer operating at 300 MHz. Some of our recent ^{1}H-NMR spectra of rHbs were obtained from a Bruker Avance DRX-300 NMR spectrometer. All Hb samples were in 0.1 M sodium phosphate (in 100% H_2O) and the Hb concentration was about 4% (2.5 mM in terms of heme). The water signal was suppressed by using a jump-and-return pulse sequence [30]. Proton chemical shifts are referenced to the methyl proton resonance of the sodium salt of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) indirectly by using

the water signal, which occurs at 4.76 ppm downfield from that of DSS at 29°C, as the internal reference.

Results

Biochemical characterization of recombinant hemoglobins

Recombinant hemoglobins, rHb (α96Val → Trp), rHb Presbyterian (β108Asn → Lys, and rHb (α96Val → Trp, β108Asn → Lys), used in this work were purified and characterized by the procedures used in our laboratory [5,8,28,29]. Electrospray ionization mass spectrometry was used to determine the molecular weight of each purified rHb to insure that each one has the correct mass (i.e., the measured mass was the same as that of the expected mass based on amino acid sequence to within ±0.005%). In addition, Edman degradation was also used to assess the removal of the extra amino-terminal methionine. In all cases, there was less than 3% of the N-terminal Met left. Thus, each of the rHb used in our studies has the correct mass and heme group conformation.

Equilibrium oxygen binding properties of recombinant hemoglobins

Table 1 summarizes the oxygen binding properties of rHbs and HbA in 0.1 M sodium phosphate as function of pH at 29°C. The oxygen pressure at 50% saturation, p_{50}, is a

Table 1

Equilibrium oxygen binding results for low oxygen affinity recombinant hemoglobins and HbA in 0.1 M phosphate as a function of pH at 29°C

Hemoglobin	pH	p_{50} (mmHg)	n_{max}
rHb(αV96W)	6.33	30.4 ± 1.5	2.4 ± 0.2
	6.84	24.4 ± 1.2	2.5 ± 0.2
	7.40	12.8 ± 0.6	2.8 ± 0.2
	8.02	6.2 ± 0.3	2.7 ± 0.2
rHb Presbyterian (βN108K)	7.03	43.5 ± 2.2	2.6 ± 0.2
	7.41	24.5 ± 1.2	2.9 ± 0.2
	7.96	9.6 ± 0.5	2.8 ± 0.2
rHb (αV96W, βN108K)	6.63	58.6 ± 2.9	2.1 ± 0.2
	7.43	38.1 ± 1.9	2.1 ± 0.2
	8.20	14.9 ± 0.7	2.6 ± 0.2
HbA	6.26	20.0 ± 1.0	2.9 ± 0.2
	6.59	18.9 ± 0.9	3.0 ± 0.2
	6.79	17.2 ± 0.9	3.1 ± 0.2
	6.97	14.5 ± 0.7	3.1 ± 0.2
	7.39	8.0 ± 0.4	3.0 ± 0.2
	7.67	5.6 ± 0.3	2.9 ± 0.8
	7.99	3.9 ± 0.2	2.9 ± 0.2
	8.37	2.5 ± 0.1	2.8 ± 0.2

measure of the oxygen affinity of Hb and the Hill coefficient (n_{max}) is a measure of the cooperativity in the oxygenation process of Hb. The oxygen binding properties of rHb (α96Val → Trp) are described in our recent publication [28]. Of special interest for rHb (α96Val → Trp) is that it has an oxygen affinity and cooperativity comparable to those of HbA in the presence of 2,3-DPG. rHb Presbyterian (β108Asn → Lys) has an oxygen affinity about two times lower than that of rHb (α96Val → Trp), e.g. p_{50} = 24.5 mmHg at pH 7.4 vs p_{50} = 12.8 mmHg for rHb (α96Val → Trp), both in 0.1 M phosphate at 29°C; and the corresponding n_{max} value is 2.8 for both rHbs. Our double mutant, rHb (α96Val → Trp, β108Asn → Lys), has an even lower oxygen affinity compared to those of rHb (α96Val → Trp) and rHb Presbyterian, e.g. at pH 7.4, p_{50} = 38.1 mmHg for the double mutant. The n_{max} for the double mutant is lower than those for each of the single mutants, i.e. 2.1 vs 2.8. The corresponding p_{50} for HbA is 8.0 mmHg and the n_{max} is 3.0.

Table 2 and Fig. 2 summarize the effects of an allosteric effector, IHP, and temperature on the oxygen binding properties of Hb (α96Val → Trp), rHb (α96Val → Trp, β108Asn → Lys), and HbA in 0.1 M phosphate at pH 7.4. For HbA in the absence of IHP, p_{50} and n_{max} decrease as the temperature is decreased. When IHP is added to HbA, the oxygen affinity is decreased at all temperatures so

Table 2

Effects of 2 mM inositol hexaphosphate (IHP) and temperature on the oxygen binding properties of low oxygen affinity recombinant hemoglobins and HbA in 0.1 M phosphate at pH 7.4.

Hemoglobin	Temp. (°C)	No IHP		2 mM IHP	
		p_{50}(mmHg)	n_{max}	p_{50}(mmHg)	n_{50}
rHb (αV96W)	10	2.3 ± 0.1	2.2 ± 0.2	13.3 ± 0.7	2.1 ± 0.2
	16	4.8 ± 0.2	2.4 ± 0.2	20.8 ± 1.0	2.0 ± 0.2
	20	6.6 ± 0.3	2.4 ± 0.2	27.9 ± 1.4	2.1 ± 0.2
	25	9.8 ± 0.5	2.6 ± 0.2	36.7 ± 1.8	2.2 ± 0.2
	29	12.8 ± 0.6	2.8 ± 0.2	45.6 ± 2.3	2.3 ± 0.2
	37	21.0 ± 1.0	2.6 ± 0.2	60.7 ± 3.0	2.5 ± 0.2
rHb (αV96W, βN108K)	10	11.8 ± 0.6	1.7 ± 0.2	23.2 ± 1.2	1.3 ± 0.2
	16	17.9 ± 0.6	1.8 ± 0.2	32.8 ± 1.6	1.6 ± 0.2
	20	21.8 ± 1.1	1.8 ± 0.2	39.3 ± 2.0	1.8 ± 0.2
	25	30.4 ± 1.5	2.0 ± 0.2	54.0 ± 2.7	2.0 ± 0.2
	29	38.1 ± 1.9	2.1 ± 0.2	58.0 ± 2.9	2.0 ± 0.2
	37	48.4 ± 2.4	2.3 ± 0.2	No measurement	
HbA	10	1.3 ± 0.1	1.8 ± 0.2	10.4 ± 0.5	2.5 ± 0.2
	16	2.3 ± 0.1	2.2 ± 0.2	15.3 ± 0.8	2.6 ± 0.2
	20	4.2 ± 0.2	2.6 ± 0.2	21.1 ± 1.1	2.6 ± 0.2
	25	5.5 ± 0.3	2.8 ± 0.2	27.2 ± 1.4	2.6 ± 0.2
	29	8.0 ± 0.4	3.0 ± 0.2	32.0 ± 1.6	2.6 ± 0.2
	37	15.1 ± 0.8	2.9 ± 0.2	42.8 ± 2.1	2.5 ± 0.2

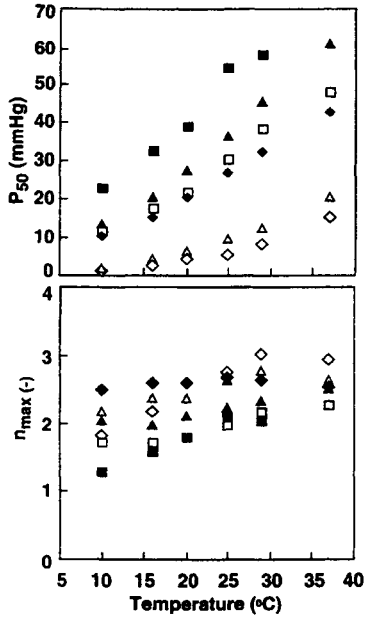

Fig. 2. Oxygen binding properties of HbA, rHb (α96Val → Trp), and rHb (α96Val → Trp, β108Asn → Lys) in 0.1 M phosphate at pH 7.4 as a function of temperature in the absence and presence of 2 mM IHP: A, p_{50}; and n_{max}. Symbols used: (◇) and (◆), HbA; (△) and (▲), rHb (α96Val → Trp); and (□) and (■), rHb (α96Val → Trp, β108AsnLys). Open symbols are for rHb samples in the absence of IHP and closed symbols are for rHb samples in the presence of 2 mM IHP.

that the overall temperature dependence of oxygen affinity is preserved. The mutant Hbs studied also show increased oxygen affinity with decreasing temperature (Table 2 and Fig. 2; ref. [28]).

^{1}*H-NMR investigations of recombinant hemoglobins*

^{1}H-NMR spectroscopy has been shown to be an excellent as well as a convenient tool to investigate the tertiary and quaternary structures of Hbs in solution [31,32]. Of special interest to this study are the exchangeable proton resonances at 14.2, 13.0, 12.3, 11.2, and 10.7 ppm from DSS, which have been characterized as the inter-subunit H-bonds in the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces in both oxy and deoxy states of HbA [33–35]. Figure 3 shows the exchangeable proton resonances of HbA, rHb Presbyterian (β108Asn → Lys), rHb (α96Val → Trp), and the double mutant, rHb (α96Val → Trp, β108Asn → Lys) in the CO form in the absence and the presence of 4 mM IHP in 0.1 M phosphate at pH 7.0 as a function of temperature. The resonance at 14.2 ppm has been identified as the inter-subunit H-bond between α42Tyr and β99Asp in the $\alpha_1\beta_2$ interface in deoxy-HbA [33], a characteristic feature of the deoxy

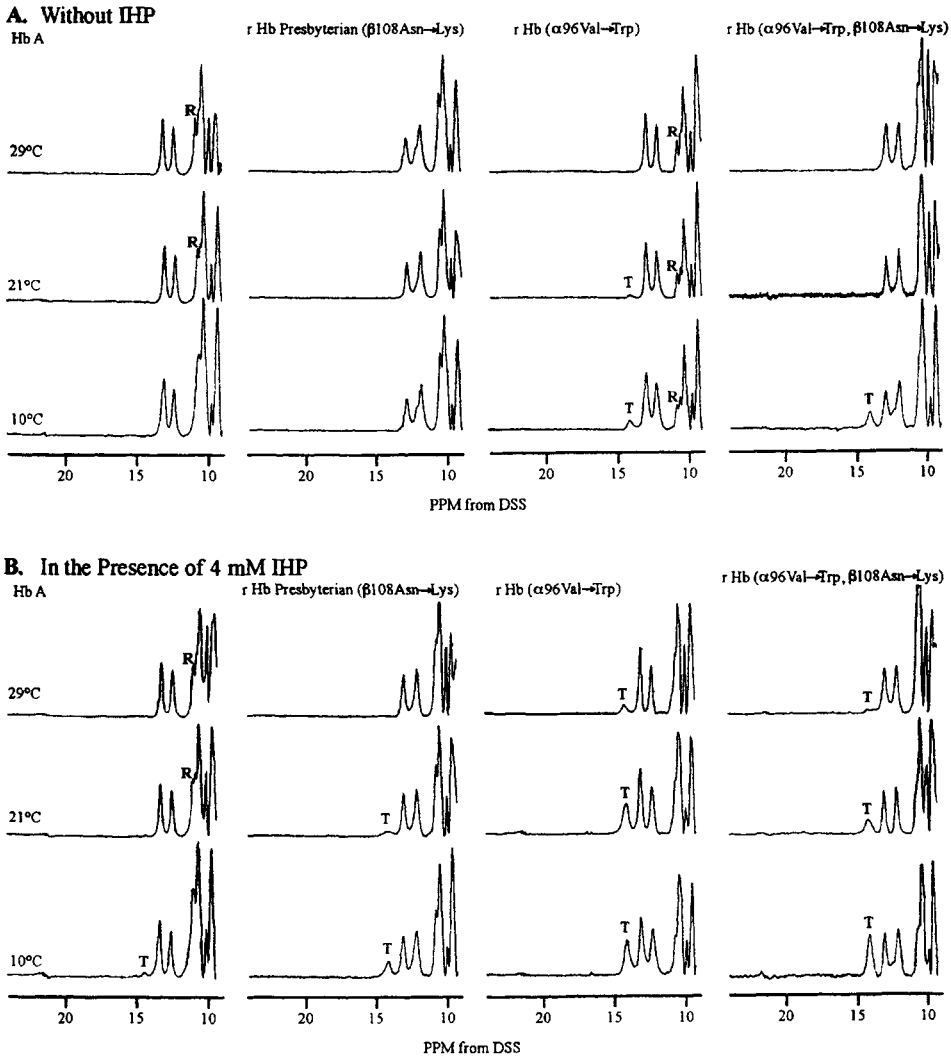

Fig. 3. Effect of inositol hexaphosphate on 300-MHz ^{1}H-NMR spectra of HbA, rHb Presbyterian (β108Asn → Lys), rHb (α96Val → Trp), and rHb (α96Val → Trp, β108AsnLys) in the CO form in 0.1 M phosphate at pH 7.0 as a function of temperature: A, in the absence of IHP and B, in the presence of 4 mM IHP. Resonances labeled R and T are characteristic markers of the respective quaternary states (see text for details).

(T)-quaternary structure of HbA [13]. In the oxy- and CO-forms, this resonance is missing [31–33]. The resonance at 10.2 ppm has been assigned to the inter-subunit H-bond between α94Asp and β102Asn in the $\alpha_1\beta_2$ interface in oxy-HbA [33,34], a characteristic feature of the oxy (R)-quaternary structure [13]. These two resonances are shown in Fig. 3. As shown for rHb (α96Val → Trp), this T-structural marker (the

14.2-ppm resonance) appears when the temperature is decreased from 29° to 10°C and/or when IHP is added to this rHb [28]. As shown in Fig. 3(A), this T-structural marker is not present in rHb Presbyterian when the temperature is decreased to 10°C, but it is present in our double mutant and rHb (α96Val → Trp) at 10°C. However, in the presence of 4 mM IHP, this T-structural marker is present in all three rHbs (Fig. 3(B)). The intensity of this T-marker is largest for the double mutant at 10°C, followed by rHb (α96Val → Trp) and then rHb Presbyterian. In HbA, there is good evidence that a signal for this T-structural marker is present at 10°C in the presence of IHP, but with a very low intensity (Fig. 3(B)).

Carbon monoxide binding kinetics of recombinant hemoglobins

The results of the kinetics of CO binding are shown in Fig. 4 for HbA, rHb (α96Val → Trp), and rHb (α96Val → Trp, β108Asn → Lys). These exhibit a time course similar to the binding of CO to a T-state Hb [36]. Table 3 summarizes the association rate constants, k_{on}, for the binding of CO to HbA and rHbs in the absence as well as in the presence of 2 mM 2,3-DPG and 2 mM IHP in 0.1 M phosphate at pH

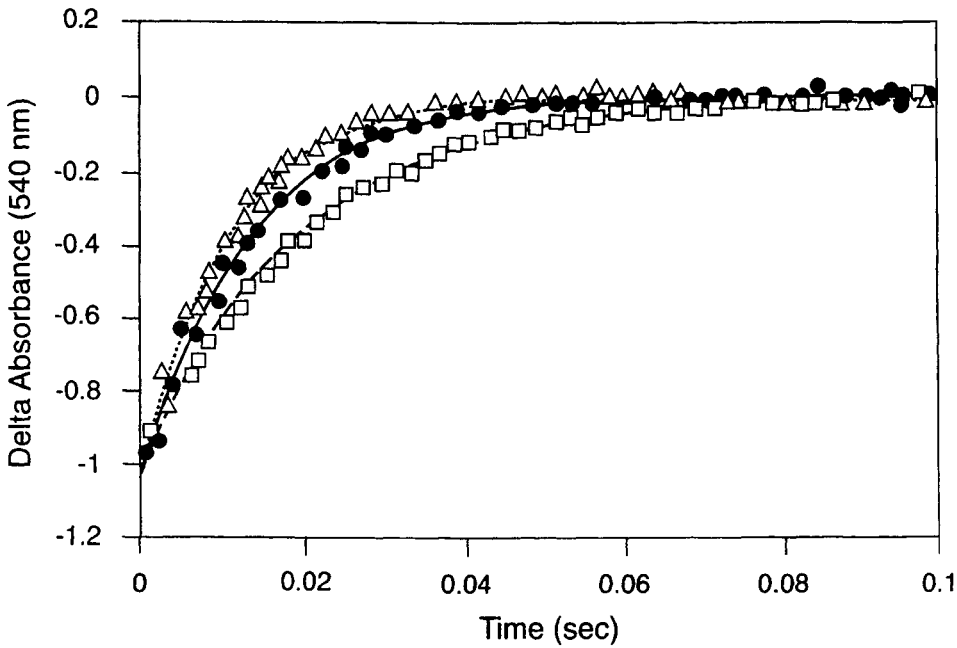

Fig. 4. The kinetics of the binding of CO to HbA, rHb (α96Val → Trp), and rHb (α96Val → Trp, β108Asn → Lys in 0.1 M phosphate at pH 7.0 and 20°C monitored at 540 nm. The final concentration of Hb was 50 μM (in terms of heme) and that of CO was 0.5 mM. Symbols used: (●), HbA; (△), rHb (α96Val → Trp); and (□), rHb (α96Val → Trp, β108Asn → Lys). The data in each case were fitted to a single exponential function.

Table 3

Effects of 2 mM 2,3-diphosphoglycerate (2,3-DPG) and 2 mM inositol hexaphosphate (IHP) on the CO association constant, k_{on} of HbA, rHbS, and low oxygen affinity mutant hemoglobins in 0.1 M phosphate at pH 7.0 and 20°C.

Hemoglobin	Association Rate Constant[a], ($\mu M^{-1} s^{-1}$)		
	k_{on}	k_{on}(+2.3-DPG)	k_{on}(+IHP)
HbA	0.154 ± 0.002^b	0.130 ± 0.005^b	0.095 ± 0.003^b
	0.144 ± 0.006^c	No measurement	0.105 ± 0.005^c
rHb S(E6V)	0.176 ± 0.005^b	0.152 ± 0.016^b	0.084 ± 0.003^b
	0.152 ± 0.003^c	No measurement	0.102 ± 0.007^c
rHb (V96W)	0.149 ± 0.007^b	0.118 ± 0.009^b	0.103 ± 0.006^b
	0.188 ± 0.014^c	No measurement	0.125 ± 0.005^c
rHb (V96W, N108K)	0.099 ± 0.002^b	0.074 ± 0.005^b	0.069 ± 0.001^b
	0.096 ± 0.005^c	No measurement	0.067 ± 0.003^c

[a]Mean ± SD (standard deviation) from several experiments.
[b]Measurements carried out at 420 nm.
[c]Measurements carried out at 540 nm.

7.0 obtained at two wavelengths, i.e., 420 and 540 nm. The k_{on} values obtained at these two wavelengths are essentially identical. The k_{on} value for HbA is $0.15\,\mu M^{-1} s^{-1}$ in the absence of IHP, in excellent agreement with the value ($0.15\,\mu M^{-1} s^{-1}$) reported by Olson [36]. The corresponding values for HbA in the presence of 2,3-DPG and IHP are 0.13 and $0.10\,\mu M^{-1} s^{-1}$, respectively. The CO association rate constants for rHb (α96Val → Trp) and rHb S (β6Glu → Val) are essentially the same as those of HbA. The k_{on} values for our double mutant are substantially lower than those for HbA and rHb (α96Val → Trp) in both the absence and presence of allosteric effectors. These kinetic data are consistent with the ^{1}H-NMR and oxygen binding results which indicate that this double mutant Hb has a very strong T-state character.

Discussion

Our approach for designing low oxygen affinity hemoglobins with good cooperativity is to make mutations in the Hb molecule that will stabilize its deoxy quaternary structure and will not disturb its oxy quaternary structure. Our rHb (α96Val → Trp) appears to achieve this goal. According to the X-ray crystallographic results for HbA [14,16,17], α96Val does not participate in any H-bonds in either deoxy- or oxy-forms in the $\alpha_1\beta_2$ subunit interface. However, the valyl side chain at α96 in the α_1-chain is closely packed against the side chain of β99Asp of the β_2-chain in the oxy (R)-form of

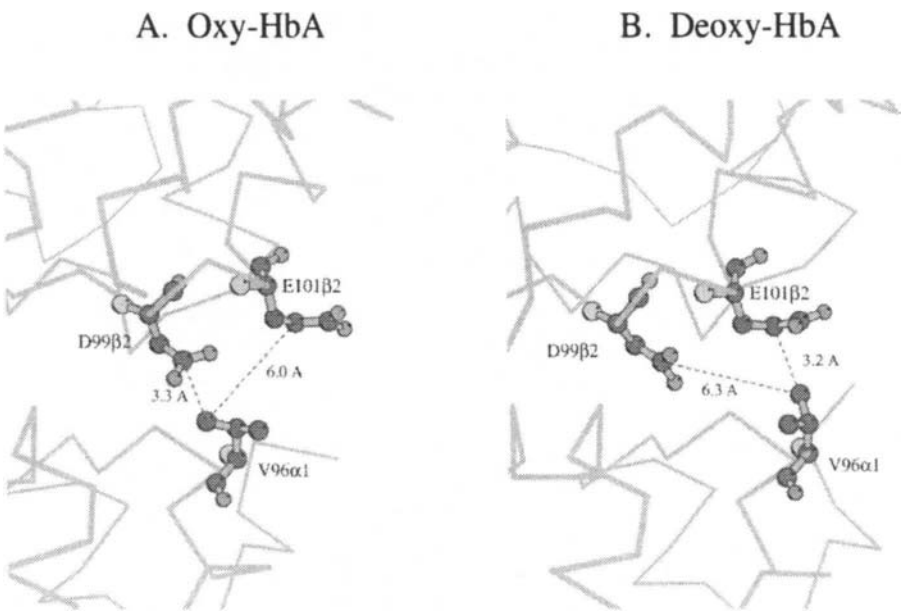

Fig. 5. Structural features of α96Val, β99Asp, and β102Glu of HbA in the $\alpha_1\beta_2$ subunit interface of oxy-HbA (A) and deoxy-HbA (B). The structures of oxy- and deoxy-HbA were obtained from the Brookhaven Protein Data Bank, entries 1HHO and 3HHB, respectively. This figure was prepared using the Molscript software package [38].

HbA (Fig. 5(A)), while in the deoxy (T) form, it is close to the side chain of β101Glu at the β_2-chain (Fig. 5(B)). Recent X-ray crystallographic investigations of rHb (α96Val $\rightarrow$ Trp) in the T-state show that the side chain of α96Trp extends into the central cavity and that α96Trp makes a water-mediated H-bond with β101Glu [37]. These structural features of rHb (α96Val $\rightarrow$ Trp) clearly suggest that the T quaternary structure of this mutant Hb is stabilized, both in the $\alpha_1\beta_2$ interface through a water-mediated H-bond between α96Trp and β101Glu and in the central cavity through the presence of a bulky side chain (i.e. Trp). This latter arrangement would also make the central cavity less able to accommodate the usual conformation for the R quaternary structure. These structural features support our ^{1}H-NMR findings that this rHb prefers to remain in the T quaternary state (as evidenced by the presence of the T-state NMR marker) even when it combines with CO [28]. In other words, this rHb in the CO form can switch its R quaternary structure to the T form without changing the ligation state by decreasing the temperature and/or by adding IHP [28]. It should be mentioned that our original molecular dynamic simulations predicted that the T structure of rHb (α96Val $\rightarrow$ Trp) is stabilized through the formation of a new H-bond between α96Trp and β99Asp. This prediction is not consistent with the recent crystal structure of this rHb. However, the main point is that the T

structure of this rHb is more stable than that of HbA. This point is supported by our experimental results as well as the new crystal structure of rHb (α96Val → Trp) [37].

Hb Presbyterian (β108Asn → Lys) is a naturally occurring low affinity mutant with high cooperativity [21–25]. Even though the oxygen affinity of rHb Presbyterian is lower than that of rHb (α96Val → Trp) (see Table 1), the T-structural marker (i.e. the 14.2-ppm exchangeable resonance assigned to the $\alpha_1\beta_2$ subunit H-bond between α42Tyr and β99Asp in the T-structure [33]) does not appear when the temperature is lowered from 29° to 10°C, in contrast to the observation for rHb (α96Val → Trp) (Fig. 2(A); ref. [28]). On the other hand, this T-structural marker appears when IHP is added to rHb Presbyterian when the temperature is decreased to 21°C (Fig. 2(B)).

To further test our working hypothesis, we have constructed and expressed a double mutant, rHb (α96Val → Trp, β108Asn → Lys). This double mutant has a lower oxygen affinity than either rHb (α96Val → Trp) or rHb Presbyterian, and still maintains a high cooperativity ($n_{max} > 2$) (Table 1). ^{1}H-NMR results indicate that the T-structural marker at 14.2 ppm appears when the temperature is decreased below 21°C, similar to that of rHb (α96Val → Trp), but its intensity is greater than that for rHb (α96Val → Trp). Also, in the presence of 4 mM IHP, the intensity of the T-structural marker is greatest for our double mutant compared to that for rHb (α96Val → Trp) and rHb Presbyterian (Fig. 2(B)). The experimental results (oxygen binding properties, CO association rate constants, and ^{1}H-NMR spectra) reported in this chapter are all consistent with the conclusion that this double mutant Hb prefers to remain in the deoxy quaternary state. Thus, it is not surprising that the Hill coefficent (n_{max}) for this double mutant is much lower than that for HbA, especially at low temperature such as at 10°C and in the presence of IHP (Fig. 4 and Table 2). Under these conditions, this rHb greatly prefers to remain in the T state even when it is oxygenated.

The structural consequences for our double mutant, rHb (α96Val → Trp, β108Asn → Lys, as discussed above provide support that we have constructed a rHb which prefers the T-quaternary structure, even when it is ligated. This rHb exhibits low oxygen affinity and high cooperativity in the oxygenation process. We plan to extend our approach to design additional low affinity mutant Hbs and to carry out additional structural and computer simulation studies of our mutant Hbs in the hope of gaining further insights into factors affecting oxygen affinity, cooperativity, and stability of the hemoglobin molecule. Hemoglobins with low oxygen affinity and high cooperativity are potential candidates for Hb-based oxygen carriers. In the future, recombinant hemoglobins with varying degrees of oxygen affinity may be designed and applied to specific medical conditions of patients who may have specific requirements for oxygen delivery, such as in cardiac surgery, natural disasters, battlefields, therapeutic treatments, etc. These new Hb-based oxygen carriers may serve as the second- or third-generation blood substitutes.

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 23

Properties of Poly(ethylene glycol)-conjugated Red Blood Cells

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Introduction

Poly(ethylene glycol) (PEG) is an amphiphilic linear polymer, which is non-immunogenic, non-toxic and chemically inert in biological systems [1,2]. Twenty years ago, Abuchowski et al. [3,4] developed a technique to modify proteins by the covalent attachment of PEG, using bovine serum albumin as a model protein. The PEG-modified albumin was shown to be intrinsically less immunogenic and to have a prolonged circulation time in rats that had been pre-sensitized with unmodified bovine albumin [3]. The covalent attachment of PEG is now commonly used to modify many proteins, enzymes, drugs and artificial surfaces [1,2,5]. PEG-modified enzymes are in clinical use, e.g. PEG-adenosine deaminase for the treatment of severe combined immunodeficiency syndrome [6,7]. PEG-modified hemoglobin has been developed for use as a blood substitute [8,9], and liposomes coated with PEG have been evaluated for use both as artificial hemoglobin carriers and as drug delivery systems [10–12].

Despite these seemingly diverse applications, there are essentially two reasons for modifying a protein or other substrate with PEG. The first, as reported by Abuchowski et al. [3,4] is to camouflage a potentially antigenic protein or enzyme from the immune system. The reduced antigenicity is believed to derive from the extremely hydrophilic nature of the PEG molecule, which in aqueous solution is surrounded by a large volume of coordinated water. Once attached to a protein near to a potentially antigenic site, the PEG molecule with its associated hydration sphere sterically hinders the approach of antibodies or other proteins [11,13]. The second reason for PEG-modification is to alter the physical properties of the substance to which the PEG is attached. For example, in PEG-modified bovine hemoglobin, aside from reducing the potential antigenicity of the bovine protein, the PEG increases the molecular weight of the hemoglobin, which slows the clearance and reduces renal toxicity [9]. In the case of PEG-modified liposomes, the PEG-coating increases the hydrophilicity of the surface, preventing aggregation (flocculation) of the particles in

suspension, and significantly delays uptake by the reticuloendothelial system [14]. Covalently bound PEG is also used to modify plastic surfaces to improve biocompatibility [1,15], and to solubilize pharmaceutical agents that would otherwise be too hydrophobic for use [1].

Over the past three years, we and others [16–19] have developed techniques to covalently bond PEG onto the surface of normal, viable red blood cells (RBC). When bound to the RBC surface, the PEG molecules and their associated water form a coating analogous to a thin layer of hydrogel. This creates a steric barrier, which prevents large molecules, such as plasma proteins, from reaching the RBC surface. However, because the barrier is comprised mostly of water, small molecules (e.g. sugars, amino acids) and dissolved gases can freely diffuse through the PEG layer, thus the function and viability of the RBC should not be compromised.

The PEG coating of red blood cells has two significant effects. Firstly, it makes the RBC surface less accessible to antibodies, and therefore "masks" the blood group antigens. Masking of the RBC blood group determinants by PEG bound to the RBC surface has the potential to circumvent many of the problems encountered in blood transfusion. Secondly, it reduces or abolishes RBC-RBC aggregation by preventing the interaction of fibrinogen with the RBC surface, which results in a dramatic reduction in blood viscosity at low shear rates. Reduction of blood viscosity by this means may be preferable to existing alternatives for the management of acute or chronic ischemia. Both of these potential applications are discussed later in detail.

PEG-coating of red blood cells

For use in a clinical setting, the PEG-coating technique should ideally be inexpensive, rapid and simple to perform, and should cause no damage to the RBC. With these criteria in mind, we identified suitable PEG-derivatives and developed and optimized the PEG-coating technique as follows.

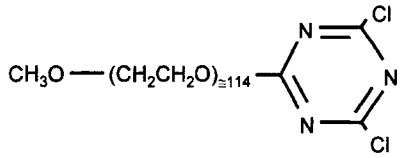
PEG derivatives

To enable conjugation with proteins, one or both ends of the PEG molecule must be modified by the addition of a chemically reactive functional group, which acts as a "linker" to bind the otherwise inert PEG molecule to the target protein. A wide range of different functionalized PEG derivatives have been synthesized for this purpose, many of which are commercially available. Most of these derivatives react primarily with amino groups (lysine residues) [1,2,5]. These PEG derivatives vary in terms of their reactivity and their optimal reaction conditions. To modify RBC without loss of viability, it was necessary to find a derivative that could be used in aqueous media under conditions as near to physiological as possible. Twenty different monofunctional PEG derivatives, each of 5000 D molecular weight, were incubated with washed RBC suspended in triethanolamine buffer at pH 8.6 for 2 h at 25°C. The RBC were then washed and resuspended in autologous plasma. Successful PEG-coating of the

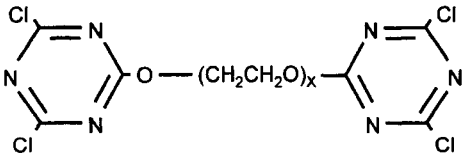
cell was assessed by looking for inhibition of RBC aggregation, using an automated RBC aggregometer and by visually examining the rate and extent of rouleaux formation after a well-mixed sample was introduced into a chamber between a microscope slide and a cover slip.

The result of these initial screening studies was that one derivative, methoxy PEG-4,6-dichloro-1,3,5-triazine (obtained from Sigma Chemical Co., St. Louis, MO) was found to be considerably more effective than any of the others. PEG-dichlorotriazine derivatives react rapidly in aqueous media at alkaline pH with the amino groups of lysine residues and may also react with thiol groups [5]. The *N*-hydroxy-succinimidyl derivatives, mPEG-SC and mPEG-SPA (Shearwater Polymers, Huntsville, AL), also showed a tendency to reduce RBC aggregation, but to a much lesser extent. None of the other derivatives tested, including PEG-aldehyde, PEG-epoxide, PEG-hydrazide, PEG-tresylate or PEG-maleic anhydride showed any significant effect under the given conditions.

The structure for methoxy-PEG-4,6-dichloro-1,3,5-triazine is shown in Fig. 1(a). This molecule is a mono-functional derivative prepared from PEG with one end capped by an unreactive methyl group (i.e. monomethoxy PEG, or mPEG). The derivative is prepared by reacting the hydroxyl-terminated end of the mPEG molecule with an excess of 2,4,6-trichloro-1,3,5-triazine in a non-aqueous solvent such as benzene [3,20]. The trivial name for trichlorotriazine ($C_3N_3Cl_3$) is cyanuric chloride; hence, this derivative is commonly abbreviated as PEG-CN. Note that the –CN suffix denotes the heterocycle C_3N_3, and does not refer to cyanide (–C≡N). Our initial



(a)



(b)

Fig. 1. (a) Monofunctional PEG-CN derivative (monomethoxy PEG-4,6-dichloro-1,3,5-triazine), molecular weight 5000 D; (b) di-functional PEG-CN derivatives synthesized in our laboratory. Two different molecular weights of PEG, 3350 D (X ≅ 76) and 18500 D (X ≅ 420), were used. Although di-functional PEGs can potentially crosslink two adjacent RBC, this was not observed for PEG-CN derivatives below 35000 D.

studies were performed using mPEG-CN of 5000 D molecular weight obtained from Sigma. We later synthesized two di-functional PEG-CN derivatives of 3350 and 18500 D molecular weight (Fig. 1(b)) according to the method of Abuchowski et al. [3]. Once the most effective PEG derivatives were identified, the optimal PEG coating technique was then developed.

PEG-coating technique

For all studies presented in this chapter, PEG-coating of RBCs was performed as follows. Washed RBC were suspended at 50% hematocrit in 30 mM triethanolamine buffer at pH 8.3, with 0.5 g/dl human serum albumin. The desired concentration of PEG-CN was rapidly dissolved in a small volume of buffer and added to the RBC suspension. The mixture was then rocked gently at room temperature for 30 min, after which the RBC were washed twice in buffer. For some studies, the RBC were resuspended at 40% hematocrit in autologous plasma.

Initial evaluation of PEG coated RBC

After PEG-coating, RBC showed normal morphology (i.e. biconcave discs) as evaluated by optical microscopy, except when very high PEG concentrations were used, which resulted in echinocytosis [23]. RBC deformability was measured using the Cell Transit Analyzer (CTA, ABX, Montpellier, France), which measures the time taken for RBC to deform and pass through 5 μm diameter pores [21]. The deformability of PEG-coated RBC was unchanged from control, except at high PEG concentrations and only when the morphology was also compromised. Oxygen uptake and release, measured using a Hemox-Analyzer Model B (TCS Medical Products Co., Huntingdon, PA) was found to be identical to control RBC.

RBC antigen masking

Blood group antigens

There are currently over 250 known blood group antigens, most of which are classified into 23 groups. Of these, the ABO and Rh groups are of greatest significance for blood transfusion because the A, B and Rh D antigens are the most strongly immunogenic. Antibodies to A and B are naturally occurring in the majority of recipients. Thus, it is universal practice to type the recipient for the A, B and also except in dire emergencies, Rh D antigens prior to transfusion, to ensure that compatible donor blood is given. Cross-matching is used to determine whether the recipient's plasma also contains alloantibodies to one or more of the minor blood group antigens. The presence of an antibody which recognizes the donor cells may result in a transfusion reaction, in which the transfused RBC are rapidly destroyed. Alloantibodies develop because it is impossible to obtain a perfect match between recipient and donor with each possible combination of blood group

antigens, and are most likely to develop in patients who have received multiple previous transfusions.

A major focus of our studies has been to develop a PEG coating suitable for use in blood transfusion, which would be applied to the RBC prior to transfusion to camouflage the blood group antigens. The PEG coating could be used either to prevent alloimmunization, or to protect donor red cells from existing antibodies and thereby prevent their destruction in the circulation. While any reduction in RBC antigenicity may potentially reduce the likelihood of sensitization or destruction, a priori it seems likely that a high degree of antigen blockade will be required to achieve these objectives, particularly the prevention of alloimmunization.

The extent of antigen masking that could be achieved by PEG coating was examined using the three PEG-CN derivatives described above, alone and in combination, over a range of concentrations. Standard blood bank serology techniques (agglutination testing) were used for the initial screening of antigen masking; blockade of antibody was later quantified directly by flow cytometry.

Inhibition of agglutination

Agglutination studies were conducted using a standard tube test [22], with antisera (Immucor Inc., Norcross, GA and Gamma Biologicals Inc., Houston, TX) to a selected range of antigens from the Rh and other blood groups, many of which are commonly implicated in delayed hemolytic transfusion reactions. Table 1 compares the direct agglutination observed for control (untreated) RBC and RBC incubated with PEG-CN 18500 at 20 mg/ml. The striking result was that direct agglutination, which was 3+ or 4+ for the control RBC, could be completely blocked by the PEG 18500 for each of the antisera tested. The other PEG derivatives, mPEG-CN 5000 and PEG-CN 3350, also inhibited agglutination, but were slightly less effective, typically giving a 1+ reaction (data not shown).

In contrast, it proved more difficult to block agglutination by antisera to the A and B antigens. With each of the PEG derivatives, agglutination was observed with the full strength antisera. Thus, it was necessary to prepare serial dilutions of the anti-A and anti-B reagents to compare the titers at which agglutination was prevented. The

Table 1

Direct agglutination tests, using antisera for several clinically important blood group antigens, for control (untreated) RBC and RBC incubated with PEG-CN 18500 at 20 mg/ml.

	Rh antigens					Other blood group antigens					
	D	c	C	e	E	Le^b	Jk^a	Jk^b	Fy^a	N	P_1
Control	4+	4+	4+	4+	4+	3+	4+	4+	3+	3+	3+
PEG RBC	0	0	0	0	0	0	0	0	0	0	0

Table 2

Titers (reciprocal dilutions) at which anti-A antisera no longer caused detectable RBC agglutination, for RBC coated with three different molecular weights of PEG. A high titer indicates strong reactivity. Masking of the A antigen by PEG-coating is indicated by a reduction in titer

	anti-A titer		
Concentration (mg/ml)	PEG 18500	PEG 3350	mPEG 5000
0	512	512	128
10	16	128	64
20	4	64	32
30	2	64	16
40		16	16

results of these studies are shown in Table 2. Again, the PEG 18500 proved to be the most effective; the minimum titer at which detectable agglutination was observed decreased from 512 to 2 with 30 mg/ml PEG-CN 18500. Smaller PEGs were less effective; the titer was reduced to 16 at 40 mg/ml for both PEG-CN 3350 and mPEG-CN 5000. Higher concentrations of PEG were not tried, because between 30 and 40 mg/ml the normal RBC morphology was lost, and all cells became spheroechinocytic [23] when examined in autologous plasma.

Thus, PEG-coating of RBC, especially with the larger PEG molecule, was effective at reducing agglutination. However, because the PEG-coating can directly interfere with cell-cell interaction, we were concerned that we might be observing a physical effect of the PEG layer rather than antigen masking. This issue was somewhat clarified using an indirect antiglobulin test (IAGT) which is used to amplify weak agglutination reactions and to detect non-agglutinating antibodies [24]. Results for direct agglutination tests and the IAGT for anti-D are shown in Table 3. Note that

Table 3

Direct agglutination test with anti-D, and corresponding indirect antiglobulin tests (IAGT), for RBC incubated with three different molecular weights of PEG. Note that although direct agglutination may be reduced to zero, a positive IAGT indicates that residual antigen remains exposed

	anti-D (agglutination)	anti-D (IAGT)
Control	3+	3+
mPEG 5000	1+	2+
PEG 3350	0	1+
PEG 18500	0	1–2+
PEG 18500/3350	0	0–1+

although direct agglutination could be blocked by the PEG coating, agglutination was still observed with the IAGT, indicating incomplete antigen masking. For the anti-D, PEG 3350 appeared to be slightly more effective than PEG 18500. Only when the PEG 18500 and PEG 3350 were used in combination were zero IAGT results observed, and this finding was donor-specific, i.e. RBC from some donors showed negative agglutination after PEG-coating, while others consistently showed 1+ agglutination. These results suggested that although significant antigen masking was being achieved, the masking in most samples was incomplete. However, we still could not eliminate the possibility that physical inhibition of cell-cell interaction played a role in the inhibition of direct agglutination, and possibly also in the IAGT results, because the IAGT also ultimately depends upon detection of agglutination. We therefore used flow cytometry to precisely quantitate the degree of blockade of antibody binding.

Flow cytometric determination of antibody binding

A FITC-conjugated goat anti-human IgG antibody (Sigma) was used to quantitate the amount of anti-D bound to RBC after incubation with increasing concentrations of PEG-CN 3350 and PEG-CN 18500. The control and treated RBC were incubated with the primary anti-D at a high antibody-to-cell ratio to prevent direct agglutination, washed 4 times, and then incubated with the FITC-conjugated secondary antibody, again at a high antibody-to-cell ratio. The cells were then analyzed by flow cytometry. Even with the above precautions, some agglutinates were observed for the control (non-PEG-coated) RBC, though these were typically less than 50% of the total number of events. Thus, for analysis, a gate was set using the forward and side scatter pattern to include only single RBC, and exclude agglutinates of 2 or more cells.

The effect of coating with PEG 3350 on anti-D binding is shown in Fig. 2. The first column shows the modal fluorescence (in arbitrary units) of D positive control RBC. The last column shows the background fluorescence of D negative RBC. A progressive decrease in antibody binding was observed with increasing PEG concentration, which reached a minimum of about 85% inhibition at a PEG-CN 3350 concentration of 20 mg/ml. No additional effect was observed at higher PEG concentrations.

Figure 3 shows that PEG-CN 18500 alone was less effective at blocking anti-D binding than PEG-CN 3350 at the same concentration (71% vs 85%), confirming the results seen with the IAGT. However, when the PEG-CN 18500 and 3350 were used in combination, an additive effect was observed which resulted in substantial further decrease in anti-D binding (to 96%). This combination of a large and small molecular weight PEG has given the best results to date, and typically resulted in a greater degree of masking for antigens other than D, e.g. 97.4% and 99.4% for the Rh antigens C and E, respectively.

Even with the optimal PEG combination, the maximal degree of anti-D blockade varied between 90% and 99% for four donors studied, but was reproducible for each

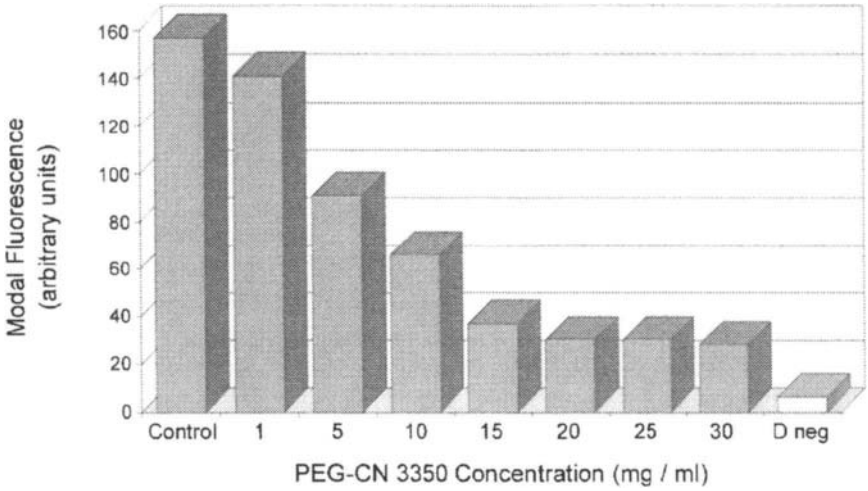


Fig. 2. Anti-D binding to RBC, measured by flow cytometry using FITC-labelled anti-human IgG: Effect of incubation of RBC with increasing concentrations of PEG-CN 3350.

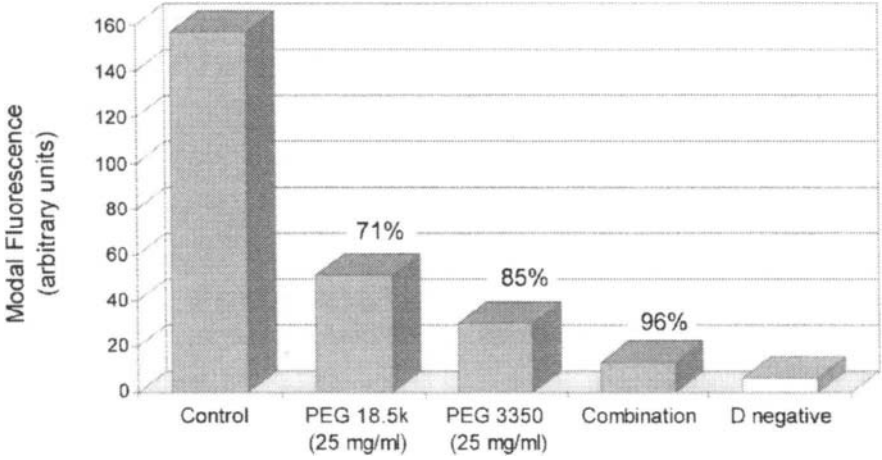


Fig. 3. Anti-D binding to RBC, measured by flow cytometry using FITC-labelled anti-human IgG: Effect of incubation of RBC with PEG-CN 18500, PEG-CN 3350, and a 50/50 mixture of both.

individual donor. The basis for this variability is not yet understood, but it is known that the number and possibly the topology of D-antigen binding sites varies with the Rh phenotype [24]. As each donor studied thus far has a different Rh phenotype, it is possible that the degree of antigen masking may be phenotype dependent. Further studies, with a larger pool of donors, are in progress to clarify this point.

Summary

We have shown that PEG-coating of RBC can very effectively prevent direct agglutination by blood group specific antibodies in the standard tube test. The larger molecule, PEG 18500, was more effective at blocking agglutination to the A and B antigens (and also the I antigen) than were the smaller PEG molecules. We speculate that this is because the A, B and I antigens are carbohydrate structures rather than proteins. These carbohydrate antigens are present in high copy number on the cell, and may be located throughout the glycocalyx at some distance from the nearest adjacent protein. As the PEG derivatives bind exclusively to proteins, the larger PEG, with its larger zone of steric hindrance, would be likely to mask more of the antigens than the smaller PEG molecules. In contrast, the IAGT and flow cytometry results showed that the smaller PEG 3350 was more effective at masking the Rh D antigen. The Rh antigens are located on transmembrane proteins, with their antigenic sites located close to the membrane. Thus, it appears that the RBC membrane surface may be more accessible to the smaller PEG molecules, while the larger PEG molecules may be excluded by steric interactions with larger RBC surface structures and the RBC glycocalyx.

It is important to recognize that the prevention of agglutination does not necessarily indicate that the RBC are "antigenically silent". This was clearly shown by the IAGT results. Thus, it was essential to demonstrate the blocking of antibody binding by techniques other than agglutination. We used flow cytometry to determine the relative inhibition of antibody binding, although an ELISA assay would also be effective, and could provide an exact measure of the number of antibody binding sites blocked.

Using the present technique, with a combination of PEG-CN 18500 and PEG-CN 3500 at 20 mg/ml, between 90 and 99% of anti-D binding can be consistently blocked, with similar or greater inhibition for the other Rh antigens. Anti-A and anti-B binding have not yet been quantitated, but are expected to be less effectively masked. The use of higher concentrations of these simple linear PEG-CN derivatives can further reduce antibody binding, but was found to severely compromise the RBC morphology. This was especially noticeable with the mPEG-CN 5000 derivative, with which RBC became severely echinocytic at concentrations above 10 mg/ml. Thus, we are currently working to develop improved PEG derivatives (i.e. with linkers other than dichlorotriazine), and to evaluate PEG molecules with a different geometry (e.g. branched and star PEGs) to create a more effective steric barrier. Our objective is to reliably mask $>99\%$ of the Rh and other minor blood antigens, with no evidence of damage to the RBC as assessed by RBC morphology and measurement of RBC deformability.

Reduction of blood viscosity

The viscosity of normal human blood is highly shear rate dependent. This non-Newtonian behavior is due to the complex interactions between RBC in the otherwise

Newtonian plasma [25]. As illustrated in Fig. 5 (upper curve), blood viscosity is lowest at arterial shear rates ($100\,s^{-1}$ and above), where the viscosity depends only upon the hematocrit, plasma viscosity and RBC deformability. However, at shear rates of $1\,s^{-1}$ and below, blood viscosity is increased because RBC begin to interact and form aggregates. At very low shear rates ($0.1\,s^{-1}$) the viscosity is typically very high (>80 mPa.s) due to the formation of large RBC aggregates (rouleaux). These low shear rates are usually restricted to the venous circulation under normal conditions. However, when normal blood flow is diminished, either locally due to vascular occlusion or globally in shock states, the disproportionate increase in blood viscosity may create a vicious cycle resulting in a worsening of ischemia [26,27].

Hemodilution, using a volume expander such as dextran or hydroxyethyl starch (HES), with or without venesection, is a well-established therapeutic option for reduction of blood viscosity, and has been shown to be effective in the management of acute or chronic ischemia, such as stroke, cerebral vasospasm, critical limb ischemia and peripheral vascular disease [28,29]. The viscosity is reduced due to a combination of the reduced hematocrit, a dilutional effect on the plasma fibrinogen concentration, and a small direct RBC anti-aggregating effect of the dextran. In general, the objective is to lower the hematocrit to between 30% and 35%, at which level the reduction in O_2 carrying capacity is offset by increased blood flow due to decreased peripheral resistance and a substantial increase in cardiac output [28]. However, hemodilution may be contraindicated or inappropriate in some disorders: e.g. myocardial ischemia (in which the cardiac reserve may be compromised), pre-existing cardiac failure, or sickle cell disease (pre-existing anemia). In such cases, an alternative means to reduce blood viscosity without significant hemodilution would be desirable. One such rheologic therapy has recently been proposed: a purified intravenous form of the PEG-poly(propylene glycol)-PEG triblock co-polymer Pluronic F68 (RheothRx™). This agent inhibits RBC aggregation and reduces low shear viscosity, and has shown some benefit in clinical trials of myocardial infarction and sickle cell crisis [30,31].

In addition to the antigen masking effects described above, the attachment of PEG also strongly inhibits the aggregation of RBC. The aggregation of RBC in autologous plasma was measured using a Myrenne Aggregometer (Myrenne GmbH, Roetgen, Germany), an instrument which quantifies RBC aggregation by measuring the rate of change of light transmission through a sheared blood sample [32]. PEG-coated RBC were prepared as described above (control RBC were incubated in buffer alone), after which both PEG-coated and control RBC were resuspended in autologous plasma at 40% hematocrit. The effect of incubating RBC with increasing concentrations of mPEG-CN 5000 is shown in Fig. 4. RBC aggregation decreased with increasing PEG concentration, and was abolished at a concentration of 5 mg/ml and above. PEG-CN 18500 was more effective, and completely inhibited RBC aggregation at 1 mg/ml (i.e. nearly 20 times more effective on a molar basis).

The effect of PEG-coating on whole blood viscosity is shown in Fig. 5, in which the viscosity of control RBC and RBC incubated with 5 mg/ml mPEG-CN 5000 was compared over a range of shear rates from $0.1\,s^{-1}$ to $100\,s^{-1}$ using a Couette

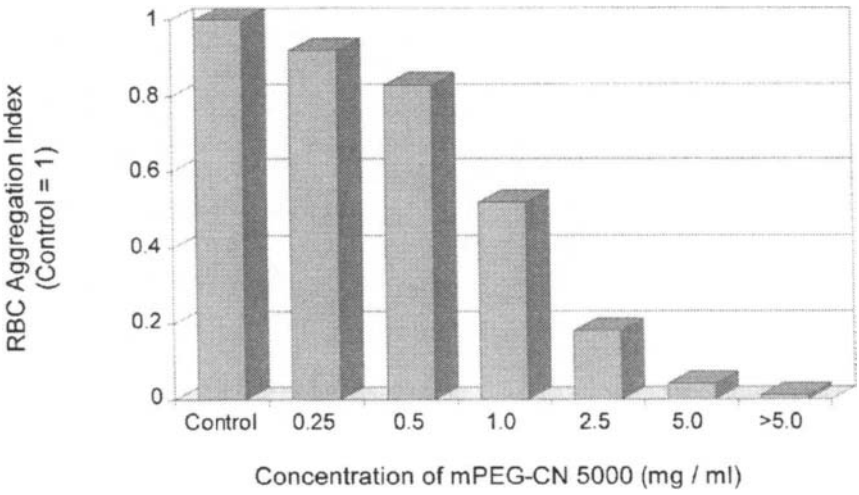


Fig. 4. Aggregation of RBC incubated with increasing concentrations of mPEG-CN 5000, measured using the Myrenne aggregometer ($n = 5$). Values are expressed relative to control (untreated) RBC.

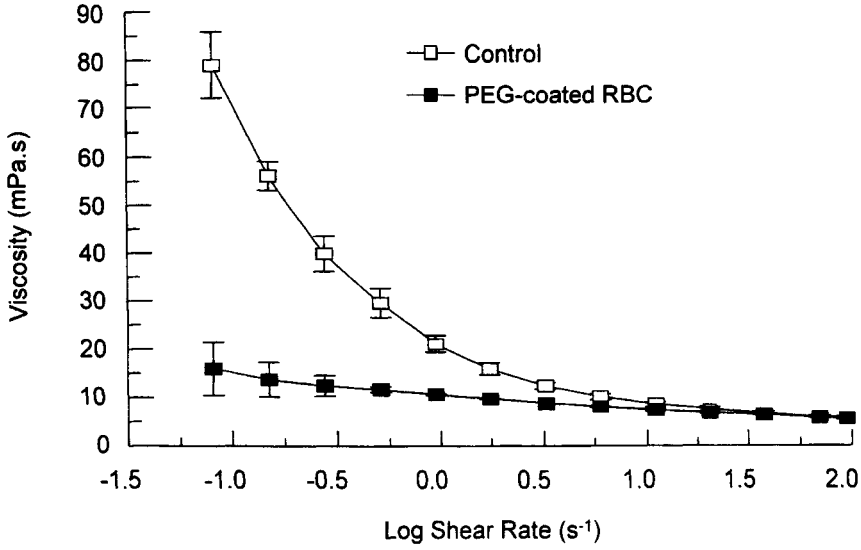


Fig. 5. Shear-viscosity relationship for RBC incubated with 5 mg/ml mPEG 5000, resuspended at 40% hematocrit in autologous plasma ($n = 5$). Compared to control (open squares) the PEG-RBC (closed squares) show a much reduced low shear viscosity but no change in high shear viscosity, which is consistent with complete inhibition of RBC aggregation.

viscometer (Contraves LS30, Contraves AG, Switzerland). The control RBC show the typical steep change in viscosity with shear rate. In contrast, the viscosity for the PEG-coated RBC (lower curve) showed very little shear dependence, i.e. the blood behaved in a nearly Newtonian manner, consistent with the complete elimination of RBC-RBC aggregation. At the higher shear rates ($10-100\,s^{-1}$), the curves for control and PEG-coated RBC exactly converged. Given that the hematocrit and plasma viscosity were identical for the two samples, this confirms that the RBC deformability was not altered by the PEG-coating. Note that the amount of bound PEG required to abolish RBC and minimize low shear viscosity is much lower than that required for antigen masking, and is therefore less likely to impair the function or compromise the survival of the RBC in any way. This would be an essential requirement for non-transfusion-related applications.

To predict the potential of PEG-coated RBC as a rheological treatment, the following questions were addressed.

How effective is PEG-coating relative to existing rheologic therapies?

In Fig. 6, the viscosity reduction that can be achieved in vitro by either hemodilution or the addition of Pluronic F68 (gift of BASF, Parsippany, NJ) is compared with that

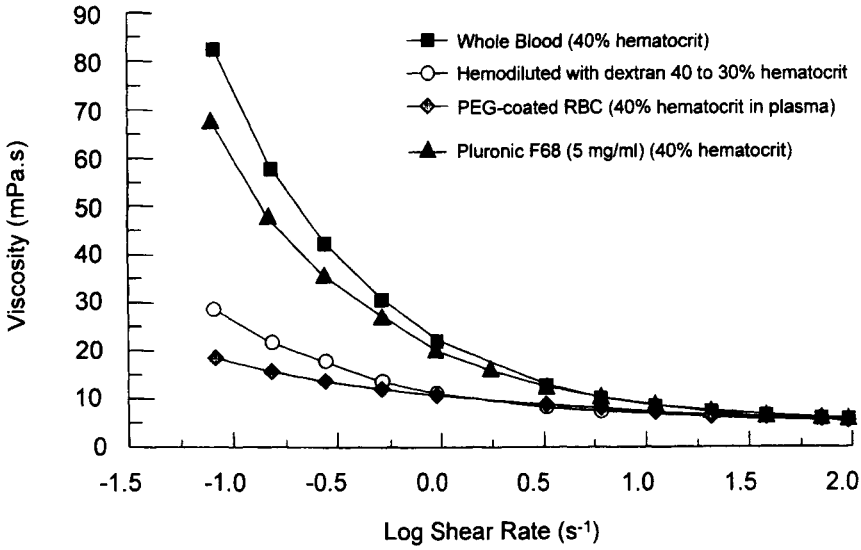


Fig. 6. Comparison of the viscosity-reducing effects of PEG-coated RBC (diamonds), Pluronic F68 (triangles) and hemodilution (circles), compared to control blood (squares). The PEG-coated RBC were incubated with 5 mg/ml mPEG-CN 5000, and resuspended at 40% hematocrit in autologous plasma. Pluronic F68 was added to whole blood at 40% hematocrit to give a final plasma concentration of 5 mg/ml. An in vitro simulation of hemodilution was achieved by dilution of whole blood to 30% hematocrit with 2% dextran 40 in saline. Note that PEG-coated RBC exhibited a lower low-shear viscosity at 40% hematocrit than whole blood hemodiluted to 30% hematocrit.

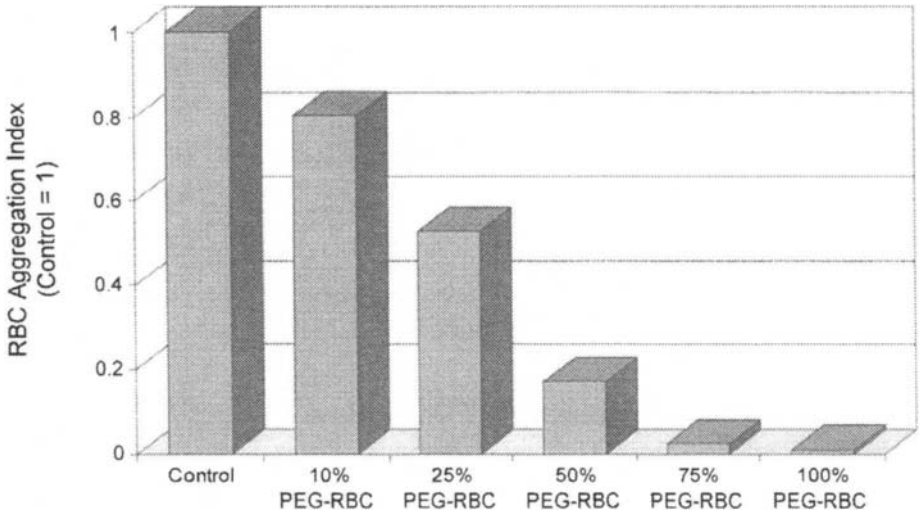


Fig. 7. Aggregation of mixtures of untreated and PEG-coated RBC in various proportions at 40% hematocrit, measured using the Myrenne aggregometer ($n = 5$). Values are expressed relative to control, i.e. 100% untreated RBC.

for PEG-coated RBC. As anticipated, hemodilution from 40% to 30% hematocrit with dextran 40 (Sigma) resulted in a large drop in viscosity, especially low-shear viscosity. However, the low-shear viscosity was still lower for the PEG-coated RBC suspension at 40% hematocrit, in 100% plasma, than for the hemodiluted blood at 30% hematocrit. Thus, the PEG-coating achieved a greater reduction in viscosity, but without compromising the oxygen-carrying capacity of the blood. In contrast, F68 caused only a modest reduction in low shear viscosity at a concentration of 5 mg/ml plasma, which represents the upper limit of the concentration that would be achieved in clinical use [33].

How many PEG-coated cells must be infused for rheologic improvement?

As the present technique involves ex vivo RBC modification, it would be difficult in practice to treat all of the circulating RBC. The proportion of treated cells required to achieve a significant rheologic effect is shown in Fig. 7. When half of the RBC were treated, aggregation was reduced by >80%, while replacement of just one tenth of RBC with PEG-treated cells reduced aggregation by 20%. This suggests that significant viscosity reduction could be practicable under clinical conditions.

Conclusions

Although this technique is at an early stage, these initial results are very encouraging. We are optimistic that, with further development, PEG-coated RBC may prove to be

a very valuable tool both for blood transfusion and as a supportive treatment in the management of acute and chronic ischemia.

Potential uses for PEG-coated RBC in blood transfusion

If the PEG-coating technique can eventually be developed to the point where all RBC blood group antigens can be effectively masked, then it could, in principle, be used to modify all units of RBC before transfusion. These "Universal Donor" blood cells could be transfused without the need for typing or matching. However, at least in developed countries, serological techniques are well established, relatively simple to perform, and the great majority of blood transfusions are entirely uneventful. Thus, the costs associated with the additional processing required to PEG-coat all transfused RBC would probably outweigh the benefits.

There are specific applications, however, in which PEG-coating may have great potential: To prevent the development of alloantibodies in patients receiving chronic transfusion therapy, e.g. sickle cell disease and thalassemia patients, who are at a high risk of alloimmunization [34,35]; to protect transfused RBC in patients with existing multiple alloantibodies, when fully matched blood cannot be located in time, or to prolong the survival of autologous RBC in patients with autoantibodies; for use in emergency transfusions, when these is insufficient time for typing and matching. In this case PEG-coated O positive RBC could be stored and used in place of O negative blood. A further advantage of this application is that the other minor blood group antigens would also be masked. For emergency transfusion, hemoglobin-based or perfluorocarbon artificial blood substitutes are also a possible alternative to O-negative blood. Blood substitutes have no antigens, and therefore require no typing and matching. However, PEG-coated RBC could have advantages over blood substitutes: Their lifetime in the circulation should be weeks rather than days; the hemoglobin will be maintained in a functional state by the normal RBC metabolic pathways; and cell-encapsulated hemoglobin will not extravasate, which is a potential cause of toxicity [36]. One proposed benefit of blood substitutes is their low viscosity, which may improve blood flow in ischemia, but this is also a feature of PEG-coated RBC. The only disadvantages of PEG-coated RBC relative to artificial blood substitutes is that the RBC will still need to be screened for infectious agents after donation, and will still require refrigerated storage. However, these are both well-established procedures, which present no particular disadvantages under most circumstances.

PEG-coated RBC for the treatment of acute and chronic ischemia

These studies have also shown the feasibility of improving blood rheology by direct modification of the surface properties of red blood cells. PEG-coating of RBC results in a similar degree of viscosity reduction to hemodilution, but with no decrease in hematocrit, and without the addition of polymer to the plasma, which can potentially cause allergic reactions and renal failure [37]. The main difference is that the PEG-

coating only affects low shear viscosity, while hemodilution also reduces high shear and plasma viscosity. Whether the reduction of high shear viscosity is beneficial in ischemia is uncertain. However, reduced plasma viscosity has recently been shown to compromise functional capillary density in animal models of hemodilution [38].

PEG-coating could potentially be useful in the treatment of ischemia from any cause. However, as there need be no reduction in hematocrit, it may be especially appropriate for disorders in which the increased cardiac output demanded by hemodilution cannot be met, e.g. myocardial infarction, shock due to hypovolemia or sepsis, and sickle cell vaso-occlusive crisis. A further advantage over hemodilution is that the treatment would be expected to last for up to several weeks rather than 1–2 d, and thus could be used to improve blood flow in patients with peripheral vascular disease.

Future studies

While PEG-coating of RBC appears to be a promising technique, our studies are at a very early stage. Before much more progress can be made, it will be necessary to determine how much PEG is bound to the RBC surface, and to which proteins it is attached; these studies will require labelled PEG derivatives. Once the PEG-modified RBC proteins have been identified, it will be necessary to determine whether their function is impaired, and if so whether there are likely to be any adverse consequences for RBC survival or potential toxicity to the recipient. The next step will be to evaluate the in vivo survival of PEG-coated RBC in animal models. Two groups have already performed some in vivo studies in mice and have found conflicting results: one group [19] showed almost normal survival for PEG-CN treated RBC, while the other group [18], using a different PEG derivative and different technique, reported poor survival (< 2 d). Some cross-species transfusion studies have been also performed [19], and the PEG coated cells were shown to have good survival relative to untreated RBC. However, more realistic studies of the residual antigenicity and the degree of protection afforded PEG-coated RBC will require an animal model with well-defined RBC blood group antigens for which antibodies are available.

Acknowledgment

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 24

Oxygen-Transport Albumin: A New Hemoprotein Incorporating Lipidhemes as a Red Cell Substitute

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Introduction

In mammals, serum albumin is synthesized in the liver and possesses a half-life in circulation of ca. 19 d. As the most abundant protein in the circulating system and with a typical concentration of $5\,g\,dl^{-1}$, it contributes 80% to the colloid osmotic pressure. The most outstanding property of serum albumin, on the other hand, is to transport an incredible variety of endogenous and exogenous compounds. Serum albumin furthermore performs many other functions as well, such as maintaining the blood pH, etc. Numerous reviews have already dealt with albumin's structure, functions and metabolism [1–5].

The albumin molecule is somewhat like a sponge. Among many substances which bind with a high affinity to albumin, the biologically important ligands are especially long-chain fatty acids and bilirubin. The interaction results in an increased solubility in plasma of both compounds. The toxicity of bilirubin is therefore decreased in this way.

Hemin released from hemoglobin is also transported to the liver by serum albumin for metabolic processing. The binding equilibria of porphyrin derivatives to albumin have been studied over the last two decades [6–11]. It has been clarified that serum albumin contains one binding site of hemin with high affinity, as well as additional sites with much lower affinity. The static spectrum of albumin-protoheme is intermediate between that of free heme and that of hemoglobin. Casella et al. clarified that the binding of the hemin to HSA involves coordination of a histidine residue of the protein to one of the iron axial coordination sites [12].

One may expect some novel functions of these albumin-heme complexes; however, little interest has been generated so far [6]. The hemopexin which is present in plasma binds free heme in both iron(II) and iron(III) forms, and the iron(II) complex definitely binds oxygen and CO reversibly, for example. Marden et al. demonstrated that the reduced deoxy human serum albumin (HSA)-protoheme complex formed a stable CO adduct and showed a typical geminate rebinding reaction after laser-flash

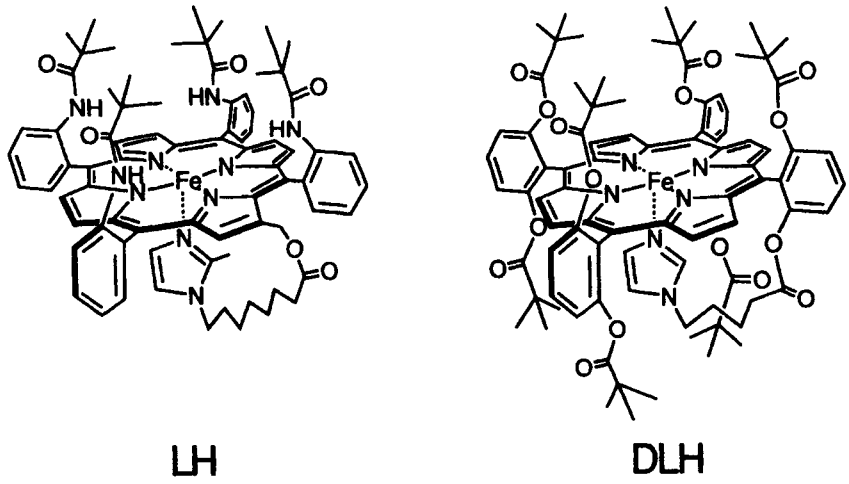

Fig. 1. Structure of lipidhemes.

photolysis [13]. Nevertheless, the oxygenated species could not be detected. Bonaventura et al. preliminarily reported the reversible spectral change of HSA with tetrakis(*o*-pivalamido)phenylporphinatoiron(II) in the presence of an excess of axial imidazole upon exposure to oxygen [14]. Their structure and the O_2-binding formation have, however, not been clarified.

We have recently found that a tetraphenylporphinatoiron(II) derivative with an intramolecular coordinated axial base, 2-[8-{*N*-(2-methylimidazolyl)}octanoyloxymethyl}-5,10,15,20-tetrakis($\alpha,\alpha,\alpha,\alpha$-*o*-pivalamido)phenylporphinatoiron(II) (lipidheme: LH, Fig. 1) [15] is efficiently incorporated into the HSA, providing a synthetic hemoprotein, which can bind and release oxygen reversibly under physiological conditions (in aqueous media, pH 7.4, 37°C) like hemoglobin [16–18]. In this chapter, the performance of this new type of O_2-carrying molecule, HSA-LH complex, as a red cell substitute are described.

Structure and ligand binding aspects of serum albumin

After numerous efforts have been made to solve the three-dimensional structure of HSA, Carter et al. reported a new tetragonal crystal that eventually proved suitable for structure determination in 1989 [19]. Furthermore, HSA was among the very first proteins crystallized in the microgravity environment in space [20]. To date, there have been several albumin structures determined by crystallographic methods. In contrast to the early conception of an oblate ellipsoid shape (140 × 40 Å), the crystal structure of HSA reveals a heart-shaped molecule which can be approximated to an equilateral triangle with sides of 80 Å and a depth of 30 Å (Fig. 2) [21]. It has been

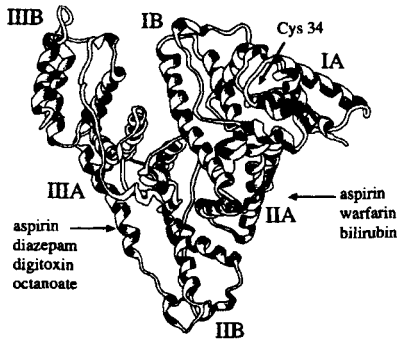

Fig. 2. Stereo view of HSA molecule (ref. [21]).

elucidated that HSA has 3 homologue domains (I, II and III) with 9 loops formed by 17 disulfide linkages, and each domain is constructed of 2 subdomains (IA, IB, etc.). The details of the HSA structure are referred to in Carter's reviews [22].

Based on an interest in the non-specific molecular binding aspect, albumin research involving binding studies has been widely developed e.g. [1]. According to the general consensus, there are six dominant regions of ligand association with albumin. The majority of the ligands are bound in one or both sites within specialized cavities of subdomains IIA and IIIA [22]. Most ligands are bound reversibly with typical equilibrium constants (K) ranging from 10^6 to 10^4 M^{-1}.

Albumin is recognized as the principal transport protein for fatty acids and other lipids that would otherwise be insoluble in the plasma. The binding constants are well-known for a variety of saturated and unsaturated fatty acids. The total fatty acid capacity of albumin varies with its chain length but averages ca six per albumin molecule. Under normal physiological conditions, one or two fatty acids are absorbed by albumin [4]. There are, however, no clear conclusions about the exact location of the bound long-chain fatty acids. A crystallographic study of fatty acid binding to HSA is now being undertaken by Carter et al.

On the other hand, it is almost accepted that bilirubin is primarily bound to a site within IIA. Concerning hemin, Hrkal et al. reported that the primary binding site is located in the sequence 124–298, which corresponds to subdomains Ib, IIa [23].

Human serum albumin-lipidheme complex as a new O_2-carrying molecule

Lipidheme binding to HSA

Synthetic tetraphenylporphinatoiron(II) derivatives have been extensively used for the research on hemoprotein models, because of their stability and advantage of covalent modification [24]. For the design of hemoglobin analogs, the proximal base bound to the central iron ion, namely the imidazole, plays a crucial role in the preparation of a stable iron-O_2 complex. From this viewpoint, a series of

superstructured porphinatoiron(II)s with an intramolecular coordinated axial base, the so-called "lipidhemes" (LHs), have been continuously synthesized by the authors [15,25]. We have recently found that LH (Fig. 1) is efficiently incorporated into serum albumin, providing a synthetic O_2-carrier, which can transport oxygen under physiological conditions (pH 7.4, 37°C) as well as in vivo [16,17].

Our first interest in this new albumin-heme molecule was its nano-structure. How many and at which place in the HSA LH molecules are bound? The HSA-LH(CO) solutions were, for example, simply prepared by mixing an ethanol solution of carbonyl-LH with an aqueous solution of HSA, followed by ultrafiltration and dialysis processes [17]. On the basis of the quantitative analysis of the free carbonyl-LH molecule in the solution prepared with different LH/HSA mixing ratios (1–14), the equilibrium constants were then calculated [26,27].

The incorporation ratios were determined to be 100% for LH/HSA: 1, 99% for 4, 94% for 8, but only 60% for 14 (Fig. 3). We concluded that the maximal binding ratio of LH to one HSA molecule was *eight*. The concentration of HSA was always constant (5 g/dl) independent of the mixing ratio. The magnitude of the binding constants for the LH association with HSA (K_1–K_8) ranged from 1.2×10^6 to 1.3×10^4 M^{-1}. These values are relatively low compared to those of palmitic acid (K_1–K_8: 6.2×10^7–3.8×10^5 M^{-1}) and hemin (K_1: 5.0×10^7 M^{-1}), which afford special interactions with albumin through their carboxylic groups. The LH molecule, on the other hand, binds the albumin only by hydrophobic interaction.

The binding sites of LH were then estimated from the binding inhibition by other ligands which occupied the major association sites of HSA. Palmitic acid,

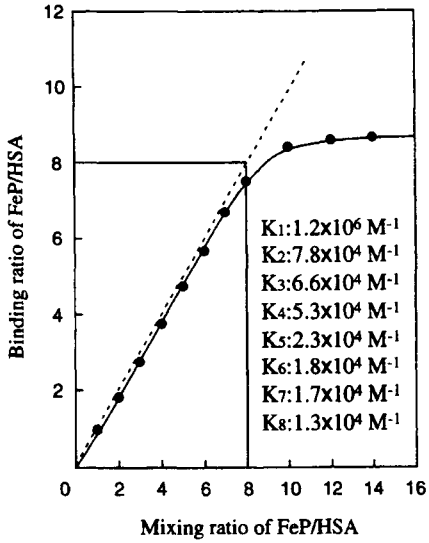

Fig. 3. Binding ratio of LH molecules to HSA.

protoporphyrin IX sodium salts instead of hemin, and phenol red instead of bilirubin were used as the inhibitors. The quantitative analysis of LH was again performed using HSA with each ligand (molar ratio: 1/1).

At an LH/HSA mixing ratio of eight, the binding numbers were significantly decreased to seven in all cases with inhibitor molecules; one of the binding regions of LH is obviously occupied by the inhibitor ligand. Consequently, the primary association sites of these ligands are identical to one of the eight binding sites of LH. It is remarkable that the LH binding was inhibited even by phenol red with a low K_I of 2.5×10^4 M^{-1}. This indicated that a minor binding site, at least a fifth one, of LH was definitely blocked by phenol red molecule.

Physicochemical property

The obtained red-colored dispersion could be stored at 4°C for three months and could be kept as a freeze-dried powder for more than six months. The aqueous redispersion of the powder does not show any differences in the turbidity, the incorporation ratio of LH, and the filter permeability.

The solution properties of HSA-LH are summarized in Table 1. Specific gravity varied from 1.013 (LH/HSA: 1) to 1.021 (LH/HSA: 8). The viscosity was the same as that of HSA (1.3 cP at a high share rate of 230 s^{-1}), which was much lower than that of human blood (4.4–5.0 cP). No aggregation was observed, even after eight LH molecules bound to one HSA.

Circular dichroism (CD) studies have provided information bearing on the secondary and tertiary structures of HSA and conformation changes that ensure when LH is bound. The spectral pattern showed typical double-minimum negative peaks in the ultraviolet region independent of the binding numbers of the LH molecules from one to eight [18]. Remarkably, there are no differences between the HSA-LH hybrid and normal HSA, suggesting that LH association does not cause any conformational changes in the albumin. The calculated α-helix content was approximately 51%, which is also the same as that of HSA itself. These observations clearly indicate that the structure of the albumin host is not affected by incorporation of LH molecules.

The binding of hemin to the specific center of the albumin molecule is generally accompanied by the rise to an extrinsic negative Cotton effect in the Soret band

Table 1

Solution property of HSA-LH solution at 37°C

System	Specific gravity	Viscosity (cP at 230 s^{-1})
HSA-LH	1.013–1.021	1.1
Serum	1.027	1.1
Human blood	1.055–1.063	4.4–5.0

region, because it binds to the albumin through a covalent bond between a histidine imidazole and the iron center, allowing a high degree of immobilization [7,12]. HSA-LH did not, however, show any CD bands at the Soret region.

The isoelectric point of HSA-LH was determined to be 4.8 from the isoelectric focusing measurement, which agreed with that of HSA. The native page also showed completely the same molecular size as HSA without LH. We concluded that the structure and surface charge distribution of HSA was not changed upon binding of the LH molecules.

O_2-binding ability

The visible absorption spectral pattern of a deoxygenated HSA-LH showed a typical five-coordinated tetraphenylporphinatoiron(II) species (λ_{max}: 439, 542, 563, 605 nm), indicating that the covalently attached imidazolylalkyl group was intramolecularly coordinated to the central iron (Fig. 4). This spectrum changed to that of the oxygenated type (λ_{max}: 423, 548 nm) upon the exposure to the air or oxygen. This O_2-association and -dissociation are reversible, and the degree of oxygenation corresponds to the O_2-partial pressure; the O_2-binding affinity [$p_{1/2}(O_2)$] was 32 mmHg at 37°C. Interestingly, the $p_{1/2}(O_2)$ values are constant independent of the numbers of binding LH molecules. The carbonyl state (λ_{max}: 424, 540 nm) was also immediately generated upon exposure to carbon monoxide gas with either the deoxy or oxy state.

The autooxidation reaction of the oxy HSA-LH (λ_{max}: 548 nm), however, took place slowly and the absorption band of 548 nm almost disappeared after 24 h, leading to formation of inactive hemin. The half-life ($\tau_{1/2}$) of the oxygenated species was 7 h at 25°C and 1 h at 37°C, respectively (under air). The activation energy of the oxidation of the HSA-LH(O_2) was calculated to be 89 kJ mol^{-1}. The $\tau_{1/2}$ was increased by raising the O_2-partial pressure (e.g. 2.3 h at 37°C in a pure oxygen atmosphere). This is the same profile as that of hemoglobin [28].

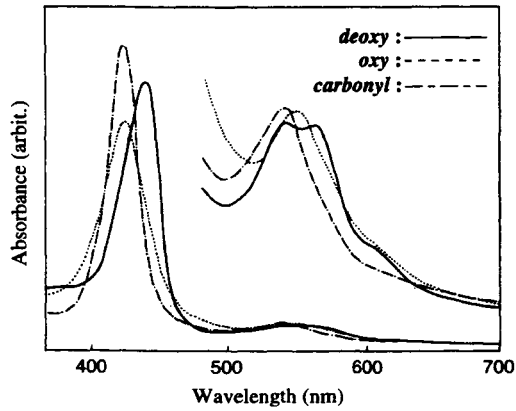

Fig. 4. Visible absorption spectral changes of HSA-LH solution.

The lifetime and $p_{1/2}(O_2)$ values of the oxygenated synthetic hemes in an organic solvent generally depend on their molecular structure and the properties of the solvent used, e.g. polarity [24]. Especially in protic solutions, the apolar cavity around the O_2-binding site is significantly effective in inhibiting an oxidation through a proton-driven process.

Although the LH molecules are incorporated into HSA by hydrophobic interaction, the micro-environment around the heme does not contribute much to the protection of the coordinated oxygen, because of its relatively short lifetime. The oxidation reaction is probably prevented mainly by its own hydrophobic substituents besides the O_2-binding site, i.e. the four pivalamide groups on the porphyrin ring. In fact, the half-life of the HSA hybrid with a double-sided lipidheme (DLH, Fig. 1) ($\tau_{1/2}$: 15 h at 25°C, 2.3 h at 37°C) was obviously longer than that of HSA-LH [18]. Consequently, the O_2-binding ability of the HSA-LH system could be controllable by modifying the molecular structure of the incorporated LH.

The O_2-binding equilibrium curves of the HSA-LH (LH/HSA: 4) were obtained from its saturated spectrum at each O_2-partial pressure (Fig. 5). Because the $p_{1/2}(O_2)$ value was ca 30 mmHg, it is expected that the HSA-LH would release 22% of the bound oxygen, if it is circulated between the lungs (pO_2: 110 mmHg) and the mixed venous system (pO_2: 40 mmHg).

The thermodynamic parameters for the O_2-binding, enthalpy changes (ΔH) and entropy changes (ΔS) for HSA-LH were estimated to be $-59\,kJ\,mol^{-1}$ and $-109\,J\,K^{-1}\,mol^{-1}$, respectively. The Hill coefficient was 1.0; no allostericity was therefore observed.

The O_2- and CO-binding parameters are summarized in Table 2. The high O_2-binding affinity of HSA-LH in an aqueous solution [$p_{1/2}(O_2)$: 14 mmHg at 25°C] compared to that in toluene (38 mmHg) mainly arises from the small O_2-dissociation constant. The CO binding parameters of HSA-LH, on the other hand, were almost the same as those in toluene. We considered that a highly polar amide-environment

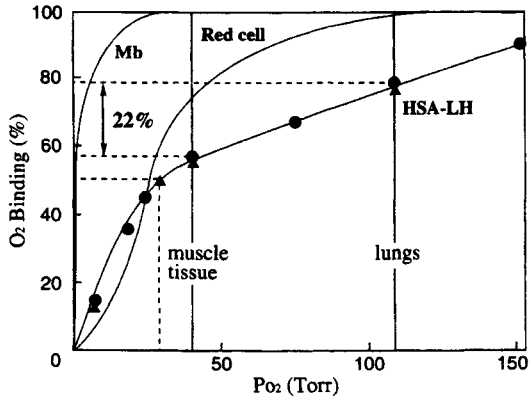

Fig. 5. O_2-equilibrium curve of HSA-LH solution at 37°C.

Table 2

O_2- and CO-binding parameters of HSA-LH solution at 25°C

System	Solution	O_2			CO		
		$p_{1/2}$ (mmHg)	$10^{-7} k_{on}$ ($M^{-1} s^{-1}$)	$10^{-2} k_{off}$ (s^{-1})	$10^{2} p_{1/2}$ (mmHg)	$10^{-6} k_{on}$ ($M^{-1} s^{-1}$)	$10^{2} k_{off}$ (s^{-1})
HSA-LH	pb[b]	14	1.9	4.3	1.4	3.9	8
r-HSA-LH[a]	pb[b]	13	1.9	4.3	1.7	3.5	9
LH	toluene	38	16	46	0.6	2.9	17
Hb (R-state)	pb[c]	0.22	3.3	0.13	0.14	4.6	0.9
Red cell	pb[b]	8.8	0.0011	0.0016	57	0.014	1

[a] r-HSA: recombinant HSA.
[b] pb: phosphate buffer (pH 7.4).
[c] pb: phosphate buffer (pH 7.0).

surrounds the LH moieties constructed by polypeptides and causes the decreasing $k_{off}(O_2)$ value. This assumption was supported by the result of a high O_2-binding affinity of LH in amide solution, e.g. $p_{1/2}(O_2)$: 0.8 mmHg in DMF.

The k_{on} and k_{off} are significantly high relative to those of red cells and hemoglobin. The experiments on the CO-binding kinetics of HSA-LH on a nanosecond time scale exhibited a rapid geminate recombination reaction, which is often observed in hemoglobin and also in the HSA-protoheme-CO complex [13].

As described above, it is quite remarkable that the molar absorption coefficient, the O_2- and CO-binding affinities, and the half-life of oxygenated species of HSA-LH are independent of the binding numbers of LH from one to eight.

From the O_2-transporting efficiency (22%) in Fig. 3, the transporting amount of oxygen was calculated as shown in Table 3. The concentration of HSA was adjusted to that in blood (5 wt%; 0.75 mM) to control the colloidal osmotic pressure. The 5 wt% of HSA-LH solution (LH/HSA: 8) can transport 3.4 ml dl^{-1} of oxygen during the circulation between the lungs (pO_2: 110 mmHg) and the mixed venouse system

Table 3

O_2-Transport by HSA-LH solution at 37°C

System	[HSA] (mM)	Heme/HSA (molar ratio)	[Heme] (mM)	O_2-transport (ml dl^{-1})
HSA-LH	0.75	1	0.75	0.42
	0.5	4	2.0	1.1
	0.75	4	3.0	1.7
	0.75	8	6.0	3.4
human blood	—	4	9.2	5.9

(pO_2: 40 mmHg). This corresponds to about 60% of the O_2-transporting amount of human blood (5.9 ml dl^{-1}), because the heme concentration of the HSA-LH solution (6.0 mM) is lower than that of blood (9.2 mM) ([hemoglobin]: 15 g dl^{-1}). The OTE value can, however, be increased by using other heme derivatives with a low $p_{1/2}(O_2)$, and the transporting amount of oxygen can be improved by increasing the HSA concentration for in vivo use.

In vivo O_2-transporting capabilities of HSA-LH solution

To evaluate the O_2-transporting capabilities of the HSA-LH, the physiological responses on exchange transfusions with HSA-LH solution in hemorrhagic shocked rats were observed. Ten male Wister rats (male, 337 ± 12 g) were anesthetized with an intraperitoneal injection of pentobarbital (50 mg kg^{-1}) and catheters (outer diameter 0.8 mmϕ), inner 0.5 mmϕ) were introduced into the right jugular vein for infusion and into the right common carotid artery for blood withdrawal. Abdominal aortic blood flow was measured as an indicator of cardiac output.

70% of the estimated total blood volume (56 ml kg^{-1}) was first exchanged with 5 g dl^{-1} HSA solution by withdrawal-infusion cycles (1 ml min^{-1}), leading to a certain decrease in the hematocrit value (Hct) of 14%. After 10 min, 40% of the circulatory volume was shed via the arterial line, affording excessively hemorrhagic rats with an Hct of only 8%. The same volume of HSA-LH solution (5 g dl^{-1}, LH/HSA: 4) was then intravenously injected, where the HSA solution was also used for relative data. Blood samples for arterial and venous blood gas analyses were taken at (i) before exchange, (ii) after 70% exchange, (iii) 10 min later, (iv) after 40% shedding, (v) after injection of HSA-LH (or HSA) and (vi) 30 min after injection. The mean arterial pressure (MAP) and renal cortical tissue O_2-tension [ptO_2(R)] were measured according to our previously reported procedures [29,30].

The MAP declined to 60% of the baseline level after 70% exchange with HSA solution and decreased to 30% immediately after shedding. The low MAP value could not be recovered simply by injection of the same amount of HSA, and all the rats died within 30 min. In contrast, the HSA-LH group showed a significant increase in MAP up to 95% of the baseline which was sustained even after 30 min (Fig. 6(a)).

Aortic blood flow was slightly increased by the initial 70% exchanging but definitely decreased to 50% after shedding. Although HSA injection tended to enhance the blood flow, the HSA-LH injection demonstrated much larger efficacy; complete recovery to the value before injection (Fig. 6(b)). The pH was somewhat decreased in the HSA-LH group but dramatically declined in the HSA group. paO_2 tended to increase even in the HSA-LH group during the experiment (max. ca 140% of the baseline), probably due to hyperventilation induced by slight acidosis.

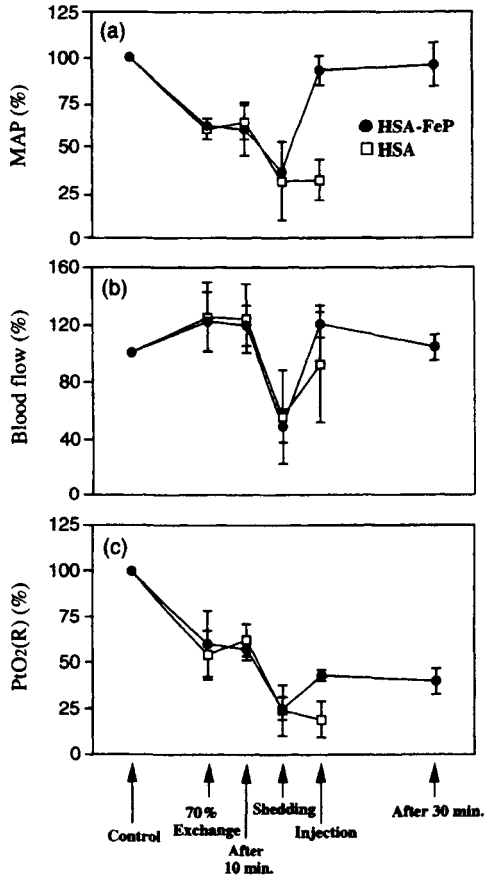

Fig. 6. Change in hemodynamic parameters during the exchange transfusion with HSA-LH solution. (a) MAP, (b) blood flow, (c) $ptO_2(R)$ shown as percentages of the basal values.

$ptO_2(R)$ generally correlates with systemic O_2-delivery, which is considered to be the product of arterial O_2-content and cardiac output. Because renal perfusion is controlled in response to a change in systemic hemodynamics, it first decreases under systemic hypoperfusion due to redistribution of systemic blood flow. Consequently, $ptO_2(R)$ is relatively sensitive to a subtle change in the systemic circulation. The monitored $ptO_2(R)$ was also obviously increased by injection of the HSA-LH solution up to 50% of the baseline (Fig. 6(c)). After the exchange transfusion, the HSA-LH group survived for more than 12 h until sacrifice, while there were significant differences in life time from the HSA group. These results are preliminary but clearly show that HSA-LH solution transports oxygen in vivo and is useful to save life at least from the hemorrhagic state.

Totally synthetic O_2-carrier with recombinant human serum albumin

Moreover, there is much current interest in recombinant HSA (rHSA) which has been recently manufactured by gene cloning and expression in *Phichia pastoris*, etc. [31]. We have already obtained several results for a rHSA-FeP complex as a totally synthetic O_2-carrier (Table 2), and a more detailed study is now being undertaken. These compounds should act as new O_2-carrying hemoprotein molecules instead of the hemoglobin in the bloodstream.

Acknowledgment

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 25

Fluorocarbon Emulsions as Blood Substitutes

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Introduction

Perfluorochemicals (PFCs) are highly fluorinated, inert organic compounds that can dissolve large volumes of respiratory gases. Liquid PFCs are colourless, odourless and non-corrosive, with specific gravities about twice that of water. PFCs are immiscible with aqueous systems, including biological fluids, but can be safely injected into the blood vasculature in an emulsified form. Emulsions containing one or more PFCs have been evaluated clinically as, for example, intravascular respiratory gas-carrying fluids (i.e. so-called "blood substitutes") and diagnostic contrast imaging agents [1–5]. Neat, unemulsified PFC liquids have also been used as respiratory tract infiltrates for the treatment of acute respiratory failure by liquid ventilation [1,5]. PFCs are not metabolised in vivo and are excreted principally as a vapour by exhalation.

This chapter gives a brief overview of progress in the development of PFC emulsions as intravascular respiratory gas carriers, focusing in particular on improved formulations and materials that have been the subject of intensive research effort during the past ca. 5 years.

First-generation PFC-based blood substitutes

The first injectable PFC emulsion that was produced commercially and tested in humans was Fluosol® (Green Cross, Japan), which contained 14.0% (w/v) of the bicyclic compound, perfluorodecalin ($C_{10}F_{18}$), and 6.0% (w/v) of the hydrofluoroamine, perfluorotripropylamine $[(C_3F_7)_3N]$ (Table 1). Fluosol® received regulatory approval for use as an oxygen-carrying adjunct to coronary balloon angioplasty in 1989–90 [1,3]. However, its use for this purpose did not live up to expectations and commercial production of Fluosol® has now ceased. The principal drawbacks of Fluosol® were:

1. its relatively low oxygen-carrying capacity, due to its low PFC content;
2. poor stability, coupled with the need for the stem emulsion component to be stored frozen,

Table 1

First- and second-generation commercial PFC emulsion blood substitutes [4]

Emulsion	PFCs	PFC concentration (% w/v)	Surfactants	Storage conditions
"First-generation" emulsions				
Fluosol®	Perfluorodecalin	14.0	Pluronic® F-68	Frozen[a]
	Perfluoro-tripropylamine	6.0	EYP[c] Potassium oleate	
Perftoran®	Perfluorodecalin	14.0	Proxanol®	Frozen[a]
	Perfluoromethyl-cyclopiperidine	6.0		
Oxypherol®	Perfluoro-trybutylamine	20.0	Pluronic® F-68	Refrigeration[b]
"Second-Generation" emulsions				
FMIQ	Perfluoromethyl-isoquinolone	50.0	EYP	Refrigeration
Oxygent™	Perfluoro-octyl bromide	60.0	EYP	Room temperature
Oxyfluor™	Perfluoro-dichlorooctane	76.0	EYP Safflower oil	Room temperature
Therox®	Bis(perfluoro-hexyl)ethene	83.0	EYP	Room temperature

[a]Stem emulsion stored frozen; annex solutions stored under refrigeration (ca 4°C).
[b]Emulsion normally stored at < 10°C.
[c]Egg yolk phospholipids.

3. its relatively short intravascular dwell time in humans (ca 7.5 h at a dose of 10 ml kg^{-1}), which was the primary reason why the emulsion was not approved for use as a treatment for anaemia [3];
4. the occurrence of acute, transient, adverse cardiorespiratory and anaphylactoid-like reactions in some patients, thought to be caused by the uptake of emulsion droplets by phagocytic cells of the monocyte-macrophage system coupled with the release of cytokines; and
5. concerns about the purity and bioreactivity of its principal surfactant constituent, the polyoxyethylene-polyoxypropylene co-polymer, Pluronic® F-68 (poloxamer 188).

Fluosol® is now regarded as a "first-generation" product, but the wealth of information obtained during the twenty years or so of research associated with its development has provided a crucial baseline from which significantly improved, second-generation, formulations have emerged.

The development of Fluosol® was paralleled by the production of other, first-generation, PFC emulsions, including Emulsion No. II and Perftoran® from China

and Russia, respectively [1–4,6]. Both formulations have similar compositions to Fluosol®, except that in Perftoran®, perfluoromethylcyclopiperidine ($C_{12}F_{23}N$) was used in place of perfluorotripropylamine to stabilise the "core" perfluorodecalin emulsion (Table 1) [6], as discussed later. In both cases, a poloxamer-type, co-polymer compound was used as sole or principal surfactant. Perftoran® has recently (1995–96) been approved for clinical use in Russia as a temporary intravascular oxygen carrier and perfusion fluid for human organ preservation and storage. A further commercial emulsion, Oxypherol®, containing 20% (w/v) of perfluorotributylamine [$(C_4F_9)_3N$], was produced by the Green Cross Corporation in Japan (Table 1), but it was never intended for clinical use because of the prolonged retention half-time of the latter compound in the body (> 500 d).

Second-generation PFC blood substitutes

The main aims of the research and development efforts to produce superior PFC emulsions which would supersede Fluosol® and other first-generation emulsions were:

1. to identify PFCs with biocompatibility and excretion properties suitable for in vivo use,
2. to improve stability characteristics, through the use of perfluorinated stabilisers and more appropriate surfactants (including lecithins and tailor-made "fluorophilic" compounds), thus producing room temperature-stable products, and
3. to develop sterilisable emulsions with significantly increased PFC content giving superior oxygen-carrying capacity (Table 2).

Such physico-chemical developments were coupled with a need for further understanding of the behaviour and efficacy of PFCs and other emulsion constituents in biological systems.

Table 2

Improvements in "second-generation" PFC emulsion blood substitutes [4]

Improvement	Significance
PFC concentration increased	Increased O_2-carrying capacity
Droplet size decreased[a]	Increased surface area for O_2 delivery
	Decreased emulsion viscocity
Improved emulsion stability	Room temparature storage
Use of EYP as surfactant	Improved biocompatibility
Use of purified Pluronic® F-68 as surfactant	Improved biocompatibility[b]
Use of "fluorophilic" surfactants	Improved emulsion stability
	Improved biocompatibility[b]

[a]Target droplet size ca 0.2 μm.
[b]Under pre-clinical evaluation.

Choice of core PFC

The two PFCs most widely studied as core constituents of injectable emulsions are perflubron (perfluoro-octyl bromide; $C_8F_{17}Br$) and perfluorodecalin (Fig. 1). The molecular weights of both compounds fall within the range 460–500 (Table 3), which is recognised as that giving acceptable tissue retention times [1–5]. It has been emphasised [7], that the excretion rate of PFCs from the body depends primarily on molecular weight, with molecular structure and the presence of cycles or heteroatoms having minimal influence. Both perflubron and perfluorodecalin can also be synthesised to a high degree of purity, thereby avoiding unwanted side-effects that have often been attributed to partially-fluorinated contaminants [5].

Other, second-generation, commercial emulsions include Oxyfluor™ (formerly Supercytes®), consisting of 76% (w/v) perfluoro-dichlorooctane ($C_8F_{16}Cl$) [8], and

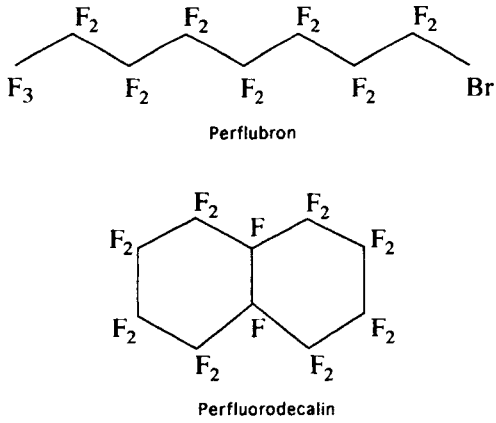

Fig. 1. Chemical structures of perflubron (upper) and perfluorodecalin (lower).

Table 3

Properties of perflubron and perfluorodecalin [1,2,4,5]

	Perflubron	Perfluorodecalin
Chemical formula	$C_8F_{17}Br$	$C_{10}F_{18}$
Molecular weight	499	462
Molecular structure	Linear	Bicyclic
Boiling point (°C)	141	142
Vapour pressure	1.87	1.67
Oxygen solubility (mmol l^{-1} at S.T.P.)	44.0	35.5
Carbon dioxide solubility (mmol l^{-1} at S.T.P.)	185	125
Critical solution temperature (°C) in *n*-hexane	−25.0	22.0
Body retention half-time (d)[a]	4	7

[a]Approximate values from animal studies.

TherOx®, containing 83% (w/v) bis(perfluorobutyl)ethene ($C_6F_{13}CH=CHC_6F_{13}$; Table 1) [2,9]. A range of second-generation emulsions containing increasing concentrations (i.e. 25–50% w/v) of perfluoromethylisoquinoline ($C_{10}F_{19}N$), together with an emulsion consisting of 54% (w/v) bis(perfluorobutyl)ethene, have also been reported [2,9]. It has been necessary to achieve a compromise between producing highly concentrated PFC emulsions with increased potential respiratory gas-carrying capacity whilst avoiding formulations with viscosities that limit their use in vivo.

A key variable in the selection of PFCs for in vivo applications is the critical temperature (°C) of solubility in *n*-hexane (CTSH) [10]. The CTSH of a PFC is a measure of its relative solubility in lipids and characterises the rate of transfer of individual compounds through alveolar membranes. PFCs with CTSH values of <28°C, such as perfluorodecalin (Table 3), are considered as lipophilic [10], whereas compounds with CTSH values >42°C, such as perfluorotributylamine, have prolonged retention times in the body. Both perfluorodecalin and, in particular, perflubron, the latter because of its molecular composition, fall into the former category (Table 3) and are thus considered acceptable for in vivo applications.

There continues to be considerable impetus in the search for other PFCs suitable for evaluation as constituents of biological gas carriers. One recent and interesting development has been the synthesis of a range of novel perfluoroether derivatives, a class of compounds hitherto considered unacceptable for use in vivo as biological gas carriers because of their generally high vapour pressures [7]. One promising compound, $HC_2F_2OCF_2C(CF_3)_2CF_2OC_2F_4H$, when emulsified with lecithin and safflower oil, had a body retention time in rats of 0.5 d [11], which is considerably less than the corresponding values for perflubron or perfluorodecalin (Table 3).

Emulsion stabilisation

As noted already, a major objective in the production of second-generation injectable emulsions has been to improve stability and, hence, shelf-life at room temperature. Emulsions are thermodynamically unstable systems and, in PFC-based formulations, the principal mechanism by which droplets grow is through a process of molecular diffusion known as Ostwald Ripening. During this process, PFC molecules from smaller droplets diffuse through the continuous phase to the larger droplets which progressively increase in size at the expense of the former [12]. Ostwald Ripening in emulsions of perfluorodecalin can be retarded by the addition of, for example, a small amount of a perfluorinated, high molecular weight, high boiling point oil (HBPO) additive, such as perfluoroperhydrophenanthrene ($C_{16}F_{26}$) [13]. This general approach was that used in the production of both Fluosol®, in which emulsified perfluorodecalin was stabilised with perfluorotripropylamine, and also in Perftoran®, where perfluoromethylcyclopiperidine was used as the HBPO [1,2,4,5]. More recently, second-generation emulsions, based on perflubron or perfluorodecalin, have similarly been stabilised against Ostwald Ripening using small quantities of an appropriate HBPO, as described later. Effective stabilisation of PFC emulsions against Ostwald

Ripening-mediated ageing has been a major hurdle for researchers to overcome in the development of room temperature-stable formulations for biomedical applications.

Perflubron-based emulsions

Perflubron has one of the highest respiratory gas-dissolving capacities of any of the PFCs commonly-used for biological applications (ca 44.0 mmol l^{-1} at S.T.P; Table 3). In addition to being a good carrier of oxygen, perflubron is also attractive for in vivo use because of its excellent imaging properties [14]. Perflubron can be readily emulsified with egg phospholipids (EYP) and shows exceptionally fast excretion characteristics (Table 3). As noted already, this is because of its very high lipophilicity due to the presence of a single bromine atom in the molecule [2,5].

Perflubron is the main PFC component in a commercial oxygen-carrying emulsion (Oxygent™) developed by the Alliance Pharmaceutical Corporation, San Diego, USA as part of a joint development effort with Ortho Biotech Inc., USA and the R.W. Johnson Pharmaceutical Research Institute (Raritan, USA), who are both subsidiary companies of Johnson and Johnson. Oxygent™ is a room temperature-stable emulsion, containing 60% (w/v) of PFC and has a shelf-life of ca 18 months. The major constituent of Oxygent™ is perflubron, but the emulsion also contains a small quantity (ca 1–2%) of its higher homologue, perfluorodecyl bromide ($C_{10}F_{21}Br$), to stabilise against Ostwald Ripening [5]. Oxygent™ has been studied extensively as an intravascular oxygen carrier in animals [1–5], and is currently being evaluated in advanced clinical trials as a temporary oxygenation fluid during surgery to reduce the need for transfusion of allogeneic (donor) blood. Multiple-site Phase II efficacy trials with Oxygent™ in patients undergoing surgery were initiated in the USA and Europe during 1995–96. Oxygent™ is, without doubt, the current front-runner of the PFC-based emulsions being evaluated as intravascular respiratory gas-carrying fluids.

The Oxygent™ emulsions are stabilised with EYP which are an obvious choice for emulsifying PFCs since they have been used extensively in injectable lipid emulsions for parenteral nutrition. EYP are excellent stabilisers of fluorocarbon emulsions, as evidenced by their ability to markedly reduce the fluorocarbon/water interfacial tension [12,15]. One problem with EYP, however, is their sensitivity to slow oxidative degradation. In some PFC-based emulsions, this has been overcome by the addition of an anti-oxidant, such as α-tocopherol, to emulsions [16]. Indeed, α-tocopherol is added at ca 0.1–0.2% (w/v) to commercial phospholipid formulations, such as Lipoid® E100 (Lipoid GmbH, Germany).

Perfluorodecalin-based emulsions

The second commonly-used PFC in second-generation emulsions is the bicyclic compound perfluorodecalin (Fig. 1). This is because its acceptable tissue retention characteristics (Table 3) outweigh its generally poor emulsifying properties [1,2,4]. Perfluorodecalin is synthesised by the progressive fluorination (using gaseous F_2) of a

hydrocarbon analogue, in the presence of cobalt trifluoride catalyst. It is produced commercially in a highly purified form by, for example, F2 Chemicals, Springfields, UK. It can dissolve ca 35.5 mmol l^{-1} of oxygen and ca 125 mmol l^{-1} of carbon dioxide at S.T.P. (Table 3).

As noted already, a range of perfluorodecalin-based, experimental emulsions, containing small (typically 0.5–2.0% w/v) quantities of a perfluorinated HBPO to retard emulsion breakdown by Ostwald Ripening [13], had biocompatibility characteristics similar to those of Fluosol® [17]. Concentrated (up to 60% w/v) emulsions of perfluorodecalin, stabilised with 4% (w/v) Pluronic® F-68 and 2% (w/v) soya oil [18], similarly had excellent biocompatibility characteristics [19].

Novel emulsions

A novel series of experimental perfluorodecalin-based emulsions, stabilised with up to 2.5% (w/v) of lecithin (Lipoid® E100, Lipoid GmbH) have been produced recently by a European team [20]. Some formulations also contained 1.0% (w/v) of perfluorodimorpholinopropane ($C_{11}F_{22}N_2O_2$) to retard droplet growth through molecular diffusion, analogous with earlier studies [13]. Perfluorodimorpholinopropane has a molecular weight of 610, a boiling point of 182°C and can dissolve ca 38.0 mmol l^{-1} of oxygen at S.T.P. It has an estimated body clearance half-time of 55 d [21]. This compares favourably with corresponding values of 60 d for the perfluorotripropylamine and perfluoromethylcyclopiperidine constituents of Fluosol® and Perftoran®, respectively [4]. The novel emulsions were prepared by homogenisation and had a total PFC content of 20–40% (w/v). Emulsions were steam sterilisable (121°C, 2 atm, 20 min), with no significant change in mean droplet diameter (ca 0.2–0.3 μm) over 300 d storage at room temperature.

Injection of male Wistar rats with 7.5 ml/kg body weight of different emulsions produced significant ($P < 0.05$), transient increases in both mean liver and spleen weights, compared to saline-treated controls, that were consistent with previous related observations [17,19]. Experiments are in progress to evaluate the efficacy of the novel emulsions as oxygen-carrying perfusates of animal organs, including both the dog heart and pig liver.

PFC emulsions as immuno-modulators

Emulsified PFCs may protect tissues, such as the coronary vasculature, against inflammatory reperfusion damage through transient alterations in blood leucocyte functions, caused primarily by uptake of emulsion droplets [22,23]. Exposure of porcine alveolar macrophages to perflubron in vitro decreases phorbol 12-myristate 13-acetate (PMA)-induced, Luminol®-enhanced chemiluminescence [24]; similar findings have been observed with human neutrophils [25]. In recent experiments, a novel emulsion consisting of 18.5% (w/v) perfluorodecalin, 1.5% (w/v) perfluorodimorpholinopropane and 2.5% (w/v) lecithin similarly produced a transient, dose-dependent, decrease in PMA-induced polymorphonuclear leucocyte (PMNL)

chemiluminescence in citrated human whole blood in vitro [20]. Chemiluminescence decreased to a maximum of 54% after 12 min ($P < 0.05$), when blood was pre-incubated with 10–40 µl of the novel emulsion, compared to that measured in saline controls. For example, the mean (± s.e.m., $n = 6$) chemiluminescence of PMNL incubated with 30 µl emulsion at 12 min following PMA stimulation (9.5 ± 1.3 mV) was significantly ($P < 0.05$) lower than that in control at the same time point (24.2 ± 2.2 mV).

A further finding from these experiments was that exposure of blood to the PFC emulsion in the absence of PMA did not induce a chemiluminescence response, suggesting that the inhibitory effects observed did not involve pre-activation of PMNL [20]. PMA is a diacylglyceol analogue which induces chemiluminescence in PMNL by direct activation of the intracellular protein kinase C (PKC) enzyme [26]. It is therefore unlikely that droplets of the novel emulsion inhibited PMA-induced chemiluminescence in PMNL by alteration of receptor-mediated activation pathways. However, other possible mechanisms include coating of PMNLs with emulsion droplets, coupled to changes in membrane function prior to subsequent uptake. This may, in turn, lead to alterations in PKC interaction with membrane-bound proteins, changes in calcium influx into the cells or direct interference of the NADPH-oxidase enzyme which is pivotal to the chemiluminescence response [24].

These studies reinforce previous suggestions [1,4] that, in addition to their use as intravascular oxygen carriers, emulsified PFCs may also be valuable in ischaemic tissues as immuno-modulating agents, acting to temporarily suppress leucocyte-mediated inflammation. Such potential anti-inflammatory effects of PFC emulsions must, however, be balanced against the potential risks of immunosuppression, especially on repeat or prolonged administration. Previous studies have shown that injection of emulsified PFCs can alter immune system function in vivo, with the responses depending on the dose, timing and route of administration relative to immunological challenge [1,27]. One objective of future work in this area should be to determine whether the composition and physical characteristics of PFC emulsions can alter PMNL functions and assess the time course and significance of any immunosuppressive responses in the recipient.

Novel fluoro-surfactants

One approach to improving the stability PFC emulsions has been to add specially synthesised, "fluorophilic" surfactants and/or co-surfactants, as pioneered by Jean Riess and colleagues [5,28–31]. The most effective compounds are those derived from sugars, amino acids and lipids. A further novel series of fluoro-surfactants, derived from glycosides (monosaccharides; "S" series) or polyols (ureas or carbamates; "P" series), have been recently produced for use in PFC emulsions. Compounds were synthesised via simple, but highly selective, routes using highly fluorinated isocyanates with amino alcohols, polyethoxylated alcohols and partially protected sugars at anomeric carbon; the overall yields were 88–95% [32]. The resultant compounds were

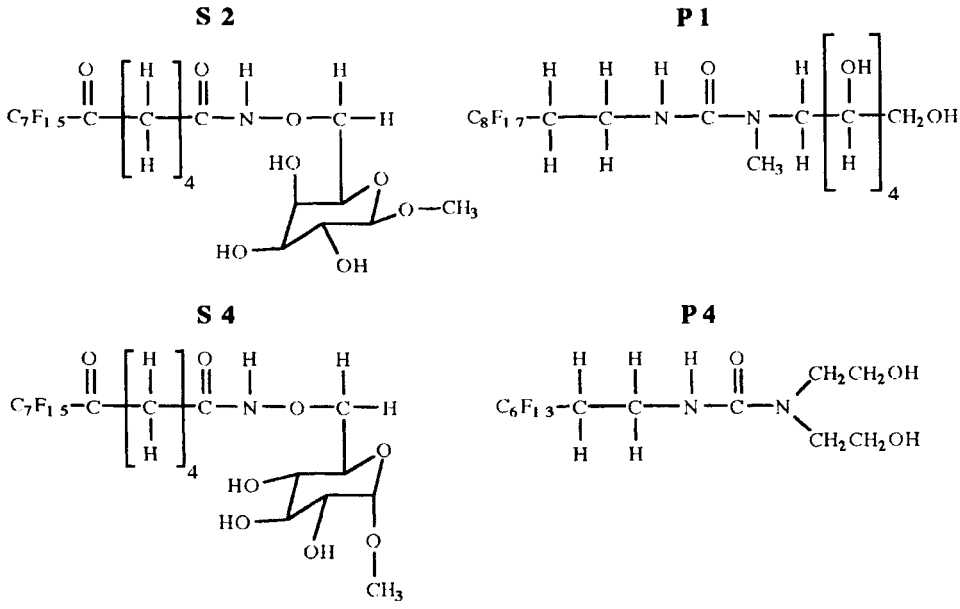

Fig. 2. Chemical structures of novel fluoro-surfactants; "S" compounds = glycoside derivatives; "P" compounds = polyol derivatives. From ref. [33].

perfluoroalkylated with hydroxylic "head" groups (Fig. 2). An interesting fluoro-surfactant to emerge from this research effort is an amphiphilic, poly(oxyethylene) monocarbamate with a general formula of $C_8F_{17}C_2H_4NHC(O)(CH_2CH_2O)_2Me$ (designated as compound P6). The biocompatibility of the fluoro-surfactants with human blood in vitro was assessed using a conventional haemolysis test. Compounds showing insignificant haemolysis at up to $10\,g\,l^{-1}$ were further evaluated for their effects on PMA-induced PMNL chemiluminescence, and in a human blood platelet aggregation bioassay [33].

Fluoro-surfactants as anti-thrombotic agents

Some fluoro-surfactants, most notably the polyol compounds, inhibited spontaneous platelet aggregation, in human blood anti-coagulated with hirudin, at concentrations of 0.01% (w/v). This suggested possible applications for these compounds as anti-thrombotic agents. In general, the effects of the fluoro-surfactants on platelet aggregation were similar to those described recently for Pluronic® F-68 [34–36]. The polyol fluoro-surfactants (e.g. compounds P1, P4 and P6), inhibited spontaneous human platelet aggregation and more closely mimicked the anti-thrombotic effects of Pluronic® F-68 than did their glycosidic-derived counterparts (e.g. compounds S2 and S4). Such inhibition of spontaneous platelet aggregation appeared to be directly related to the surfactant properties of the compounds.

It has been suggested that the beneficial effects of Pluronic® F-68 in ischaemic injury [37] may be due, at least in part, to the inhibitory effects of this compound on platelet aggregation in the microvasculature. Thus, the beneficial effects of tissue perfusion with oxygen-carrying PFC emulsions containing Pluronic® F-68 [1,4] may also involve direct effects of the surfactant on platelets. Moreover, some of the novel fluoro-surfactants, discussed above, may similarly have clinically-relevant anti-thrombotic effects, either alone or as constituents of PFC emulsions, and this aspect should be a focus of future research effort in this field.

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 26

Heparin-like New Molecules with Blood

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Introduction

Blood substitutes represent a wide spectrum of compounds ranging from platelets, white blood cells, red blood cells, to plasma proteins, lipids, carbohydrates analogous. Even if they are capable of replacing only limited and specific functions of the natural blood components, however various types of substitutes have been developed and great expectations are held for their practical use.

Blood compatibility is one of the essential requirements of any substitute introduced into the biological system. The compatibility of a polymer or any substance in blood requires that the material should not cause thrombosis, destroy cellular elements, alter plasma protein, deplete electrolytes or cause acute or delayed toxicity. Consequently, clinical researchers seek biologically active polymers which are similar to blood proteins in structure and physical characteristics.

Polymers can react with blood proteins and cell surfaces, but also with other macromolecules to form supermolecular structures. These associations occur with neutral molecules through van der Waals forms and hydrogen bonding. Polyions form even stronger electrostatic interactions through ionic linkages. Unfortunately the medical utilization of polyanions has been detered so far by their adverse toxicological effects such as anemia, leukocytosis induction, and sensitization to biological endotoxins. In human patients polyanions have produced pyrexia, thrombocytopenia and temporary blindness. However, refinement of the macromolecules such as lower M.W. and narrower polydispersity has greatly decreased these adverse effects without significantly altering their efficacy.

Since many polysaccharides are fairly compatible with most biological systems, several derivatives have been made and tested for biological applications. The first consistently effective polysaccharide utilized for medicinal purposes was heparin. Heparin is a natural anionic polysaccharide that occurs in two forms: a high molecular weight sulfate proteoglycan (HMW heparin) and a lower molecular weight heparin (LMW heparin) with a lower sulfate content (Fig. 1).

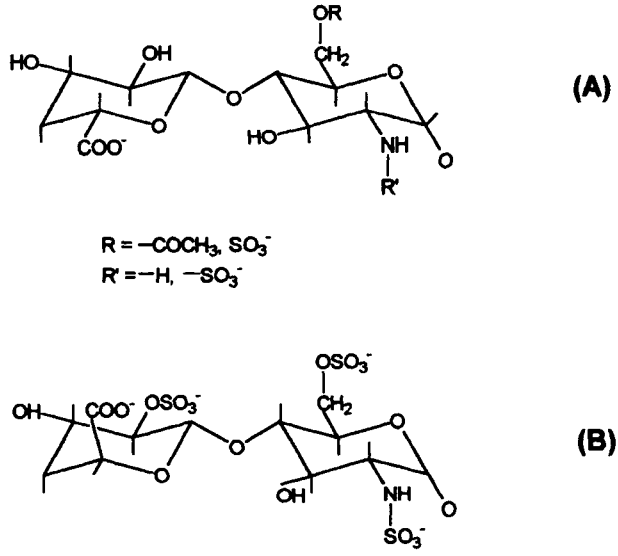


Fig. 1. Two different heparin structures: (A) High Molecular Weight (HMW) Heparin; (B) Low Molecular Weight (LMW) Heparin.

The latter, in comparison with HMW heparin, shows:

1. a greater anti-FXa activity than antithrombin one;
2. a lower platelet activation;
2. a lower capacity to stimulate fibrinolysis;
4. a longer half-life.

Other anionic polysaccharides include hyaluronate, chondroitin sulfates, dermatan sulfate and keratan sulfate. The different structures of these polymers, commonly present in mammalian tissues, are shown in Fig. 2. In contrast with the other glycosamminoglycans, the hyaluronic acid is the only one to be not synthesised as protoglycosaminoglycan chain covalently bound to a protein core. Moreover, it is constituted by a regular sequence of disaccharide units which allow us a better knowledge of the structure-property relationship.

Sulphation reaction of hyaluronic acid

Sulphated Hyal derivatives ($HyalS_x$) have been obtained by introducing sulphate groups on the OH moieties. The reaction scheme is summarised in Fig. 3 [1]. This reaction is easily controllable, so that hyaluronic acid derivatives, with different degrees of sulphation, can be synthesised simply by changing the OH mol/SO_3-pyridine mol ratio (Table 1). The final products contain from 1 to 4 sulphate groups for disaccharide unit [2] (HyalS, $HyalS_2$, $HyalS_{2.5}$, $HyalS_3$, $HyalS_{3.5}$,

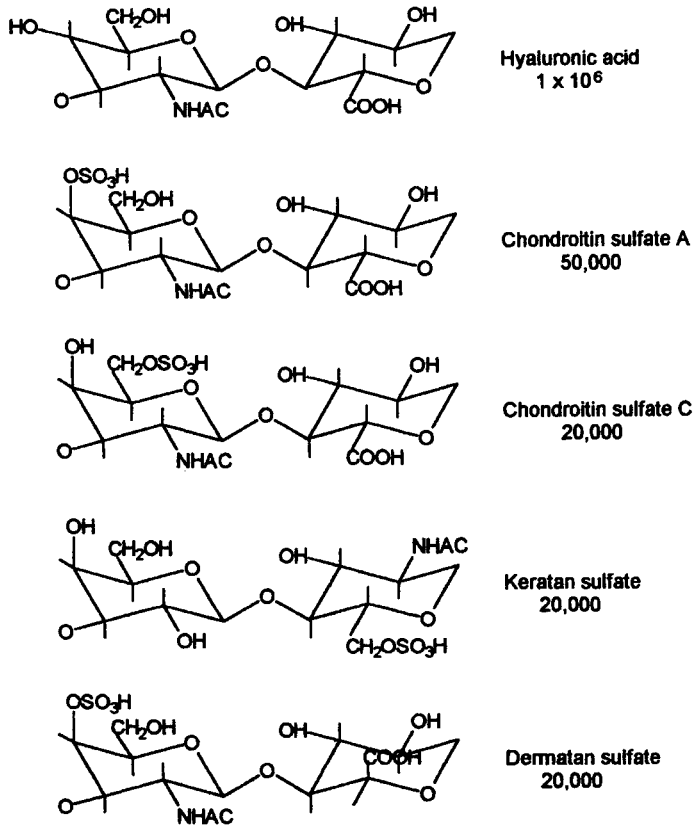


Fig. 2. Mucopolysaccharide structures.

$HyalS_{3.8}$, $HyalS_4$). The NMR analysis (see next paragraph) revealed that in all HyalSx the alcoholic group in position 6 of *N*-glucosamine ring is always esterified and in $HyalS_4$ all of the –OH groups present in the disaccharide unit (i.e. 4) are fully esterified. The other partially estherified compounds correspond to a random distribution of $-OSO_3$ groups.

Physico-chemical characterisation

Sulphated hyaluronic acid derivatives ($HyalS_x$) have been studied in terms of structural features by NMR measurements. The degree of sulphation was determinated by elemental analysis, ^1H- and ^{13}C-NMR studies. Figure 4 shows the ^{13}C-NMR spectrum of Hyal with the peaks assignment [3]. Figure 5 shows the ^{13}C-NMR spectrum of $HyalS_3$. The spectrum is characterised by a remarkable noise due to the high viscosity of the polymer. Comparing the spectra of $HyalS_3$ and Hyal is possible to evidence the low-field shift (from 61.1 to 67.7 ppm) of the glucosamine C6 signal

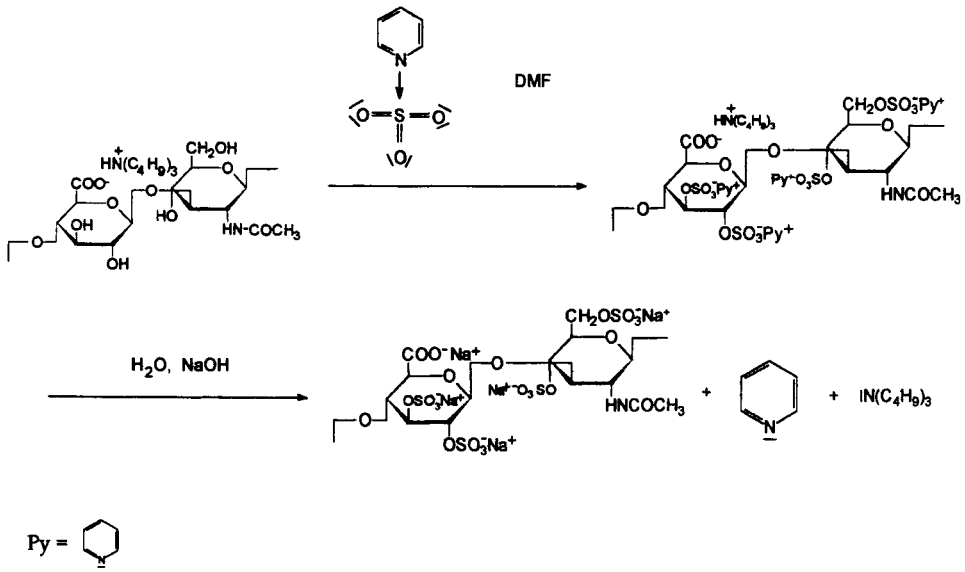


Fig. 3. Scheme of the sulphation reaction of hyaluronic acid.

Table 1

Number of sulphate groups in the disaccharide unit as a function of the OH moli/SO_3-pyridine molar ratio in the sulphation reaction

OH mol/SO_3-pyridine mol ratio	In the repeating unit sulphate groups
1:1	1
1:2	2
1:5	2.5–3.0
1:8	3.5–3.8–4.0

in the spectrum of $HyalS_3$, indicating that the sulphation in this position is complete. Such a shift was observed in other polysaccharides when a substitution with sulphated groups occurred [4].

Moreover, the signal relative to glucosamine C3, C4 and glucuronic C1 and C2 show little variations of chemical shifts and of intensity, demonstrating that a partial substitution may involve these positions.

In order to have a simplified model of hyaluronic acid, suitable for NMR studies, the tetrasaccharide β-D-Glc_pA-(1-3)-β-D-Glc_pNAc-(1-4)-β-D-Glc_pA-(1-3)-D-GlcNAc was investigated in water solution. The NOESY (Nuclear Overhauser Effect Spectroscopy) spectrum shows interesting interresidue cross-peaks between proton nuclei at distances lower than 0.35 nm. These cross-peaks are diagnostic for the

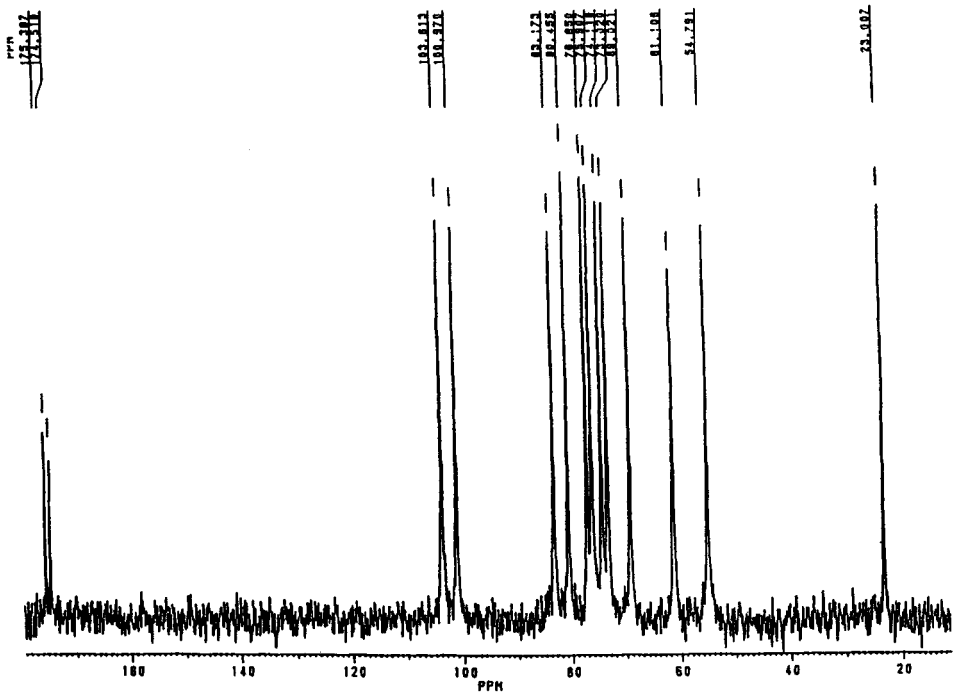


Fig. 4. ^{13}C-NMR spectrum of Hyal registered at 600 MHz.

identification of a solution structure. The most useful cross-peaks observed are: H_2 GlcNAc1-H_1 GlcA2; H_3 GlcNAc1-H_1 GlcA2; H_3 GlcA2-H_1 GlcNAc3; H_4 GlcA2-H_1 GlcNAc3; H_3 GlcNAc3-H_1 GlcA4; H_4 GlcNAc3-H_1 GlcA4. On the basis of experimental evidences an Hyaluronic acid-like structure can be inferred. Further the tetrasaccharide has then been fully sulphated.

Preliminary theoretical results obtained by Molecular Mechanics and Molecular Dynamics calculations on both tetrasaccharides molecules (the sulphated derivative and native one) point out some small differences in the molecular conformation.

The structure of the sulphated and non-sulphated tetrasaccharides are reported in Fig. 6. They show that there are non-significant conformational differences between the two polymers.

Biological characterisation of $HyalS_x$

Biocompatibility evaluation

The biocompatibility of $HyalS_x$ was evaluated in terms of cytotoxicity, cytocompatibility and hemolysis tests. The growth of the tumour cell line L929 in the presence of sulphated hyaluronic acids with different degrees of sulphation ($HyalS_{3,3.5,4}$) and at

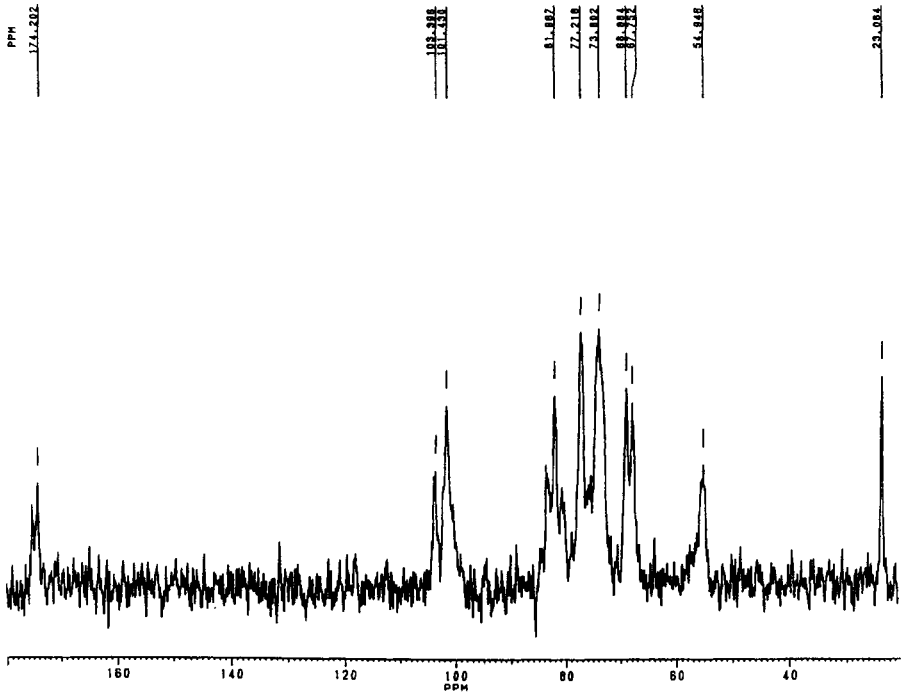
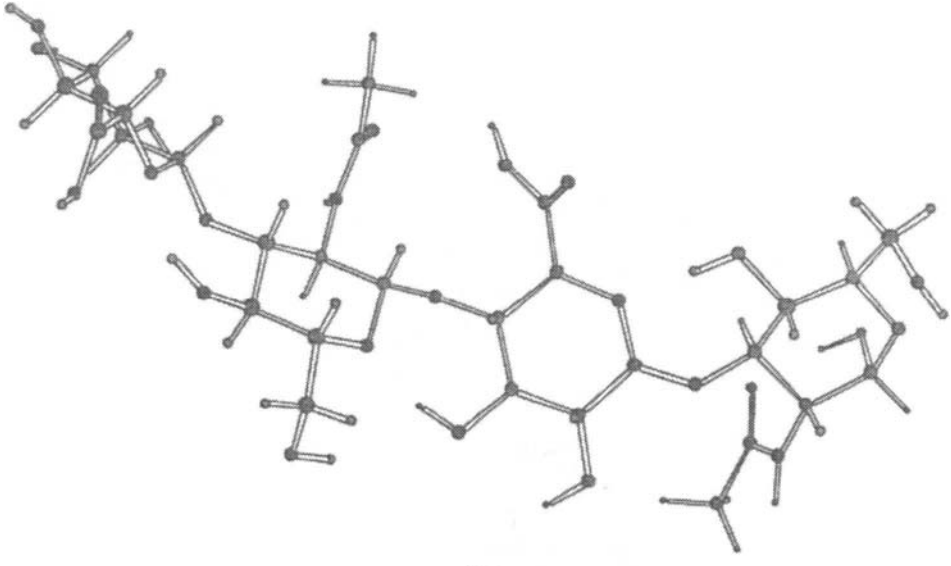
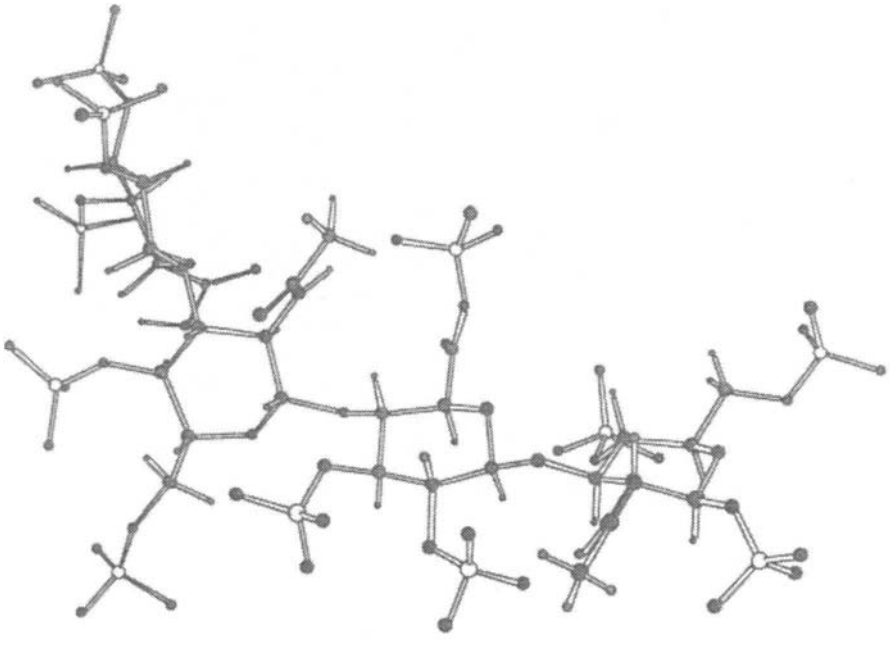


Fig. 5. ^{13}C NMR spectrum of $HyalS_3$ registred at 600 MHz.

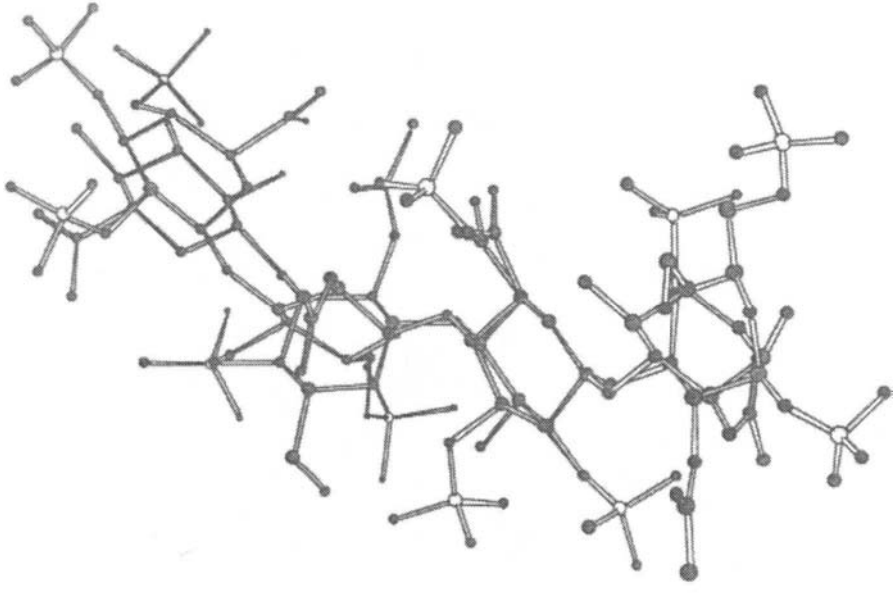


(A)

Fig. 6. (A) Hyal tetrasaccharide structure.

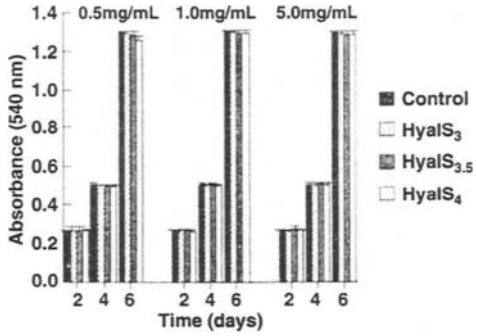


(B)

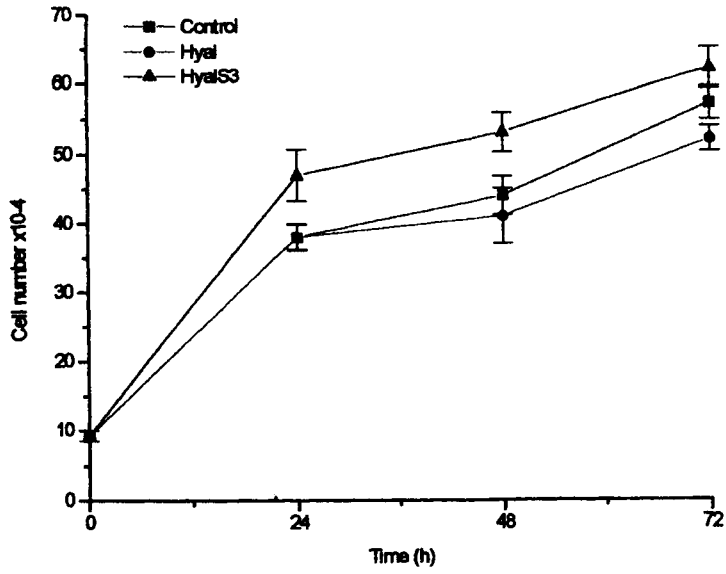


(C)

Fig. 6. (B) HyalS tetrasaccharide structure; (C) Superimposition of (A) and (B).



(A)



(B)

Fig. 7. (A) Growth of fibroblasts L929 in contact with $HyalS_x$ at three concentrations; (B) Growth of endothelial cells in contact with polystyrene, Hyal and $HyalS_3$.

different concentrations resulted not affected by both the polysaccharide concentrations and their degrees of sulphation, as shown in Fig. 7(A). In fact after 2, 4 and 6 d the cell growth was the same for the control and all the $HyalS_x$. Moreover, all the cells cultured with $HyalS_x$ showed no morphological alteration and structural changes in cell organisation.

The sulphated derivatives, as well as the non sulphated Hyal, did not affect also the growth of Human Umbilical Vein Endothelial Cells (HUVEC) [5] as shown in Fig. 7(B). The number of cells in medium containing $HyalS_x$ increased with time and a

slightly better growth was shown than in medium containing Hyal which was similar to that observed for the control. Moreover, endothelial cells in medium containing $HyalS_x$ and Hyal spread with no morphological alteration and without structural changes in cell organisation. These results demostrated a good cytocompatibility of the tested compounds. All the $HyalS_x$ derivatives were also avoid of any haemolytic effect as demostrated by the results of haemolysis test; the control curve was in fact completely superimposed upon the curve obtained from the sulphated samples.

Enzymatic degradation of $HyalS_x$

Enzymatic degradation studies were carried out to demonstrate $HyalS_x$ metabolic resistance compared with heparin which is quickly degraded in the organism by heparinase [6]. The evaluation of the enzymatic digestion [7] was estabilished by digestion of the compounds with Streptomyces hyalyronidase and analysis of the obtained fractions with SDS-Gel Electrophoresis. The not digested Hyal showed a characteristic wide band which disappeared when the digestion products were eluted with the solvent. On the contrary, the results obtained on $HyalS_{3,3.5,4}$, demonstrated that hyaluronidase did not digest these samples since the bands obtained can be superimposed upon the not digested Hyal band. These results were confirmed by Fast Flow Chromatography analysis. Infact, as shown in Fig. 8, the elution profile of Hyal changed because of enzymatic digestion, whereas $HyalS_x$ did not change before and after treatment with the enzyme. The presence of negatively charged sulphate groups determined the non attack of the enzyme.

In order to verify if sulphated hyaluronic acid derivatives may be digested by chondroitinase, $HyalS_3$ was also treated with this enzyme. The results obtained demonstrated chondroitinase, as well as hyaluronidase, was not able to digest $HyalS_x$.

Anticoagulant activity of $HyalS_x$

The anticoagulant activity of $HyalS_x$ was evaluated and compared with Unfractionated Heparin (UH) in order to understand how the sulphation degree affects the biological activity of the tested polysaccharides [2]. As demostrated by the values of the Whole Blood Coagulation Time (WBCT), the Thrombin Time (TT) and the Reptilase Time (RT) determined for the different samples (Table 2), the anticoagulant activity of Hyal derivatives was found to be strictly correlated to their degree of sulphation and to increase with increasing the number of sulphated groups per disaccharide unit. In fact, the first two compounds, i.e. HyalS and $HyalS_2$, showed a WBCT similar to that of hyaluronic acid which exhibited a time slightly longer than the control. On the other hand, when sulphated hyaluronic acids containing more sulphate groups than 2 were used, a jump in the WBCT was observed. The TT test, revealed that HyalS and $HyalS_2$ were not able to prolong the clotting time by inhibiting thrombin activity as well as Hyal, while increasing the degree of sulphation a prolongation was observed. The RT values determined for these compounds were the same as that of the control sample, excluding any possible interference of other

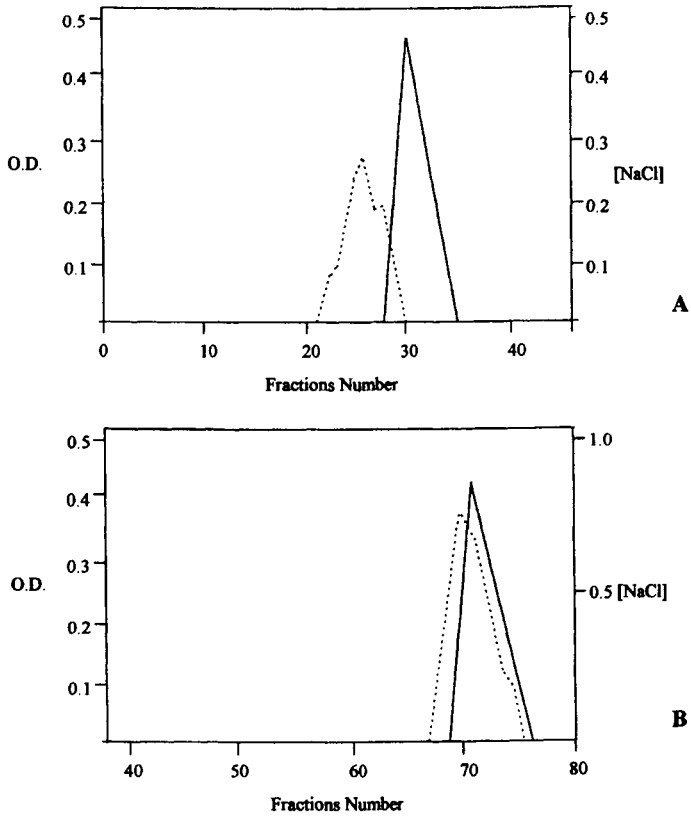


Fig. 8. Fast flow chromatography of Hyal and $HyalS_3$, treated and non treated by hyaluronidase (A) — non treated Hyal, — enzyme treated Hyal; (B) — non treated $HyalS_3$, — enzyme treated $HyalS_3$.

Table 2

Coagulation assays for the different sulphated hyaluronic acids. WBCT = Whole Blood Clotting Time, TT = Thrombin Time, RT = Reptilase Time

Sample	WBCT (min)	TT (s)	RT (s)
Control	25 ± 2	10 ± 2	14 ± 1
UH	> 120	> 120	14 ± 1
Hyal	45 ± 5	11 ± 2	14 ± 1
HyalS	40 ± 5	11 ± 2	14 ± 1
$HyalS_2$	45 ± 5	12.5 ± 1.5	14 ± 1
$HyalS_{2.5}$	> 120	30[a] ± 2	14 ± 1
$HyalS_3$	> 120	38[b] ± 2	14 ± 1
$HyalS_{3.5}$	> 120	> 120[c]	14 ± 1
$HyalS_{3.8}$	> 120	> 120[d]	14 ± 1
$HyalS_4$	> 120	> 120[e]	14 ± 1

[a] $1\,mg = 2.6 \times 10^{-3}$ mg heparin; [b] $1\,mg = 1.3 \times 10^{-2}$ mg heparin; [c] $1\,mg = 1.8 \times 10^{-1}$ mg heparin; [d] $1\,mg = 3.7 \times 10^{-1}$ mg heparin; [e] $1\,mg = 4.6 \times 10^{-1}$ mg heparin.

Table 3

Thrombin inactivation (Fibrinoformation Time) in the presence of AT III, HC II and without cofactors as a function of the degree of sulphation

Samples [1 mg/ml]	Direct thrombin inactivation (s)(± 1)	In the presence of AT III (s)(±1)	In the presence of HC II (s)(±1)
Control	14	14	14
UH[a]	15	> 120	> 120
Hyal	15	15	15
$HyalS_2$	15	15	15
$HyalS_{2.5}$	19	19	31
$HyalS_3$	25	24	36
$HyalS_{3.5}$	36	34	> 120
$HyalS_{3.8}$	45	45	> 120
$HyalS_4$	> 120	> 120	> 120
$HyalS_4$[b]	55	55	—

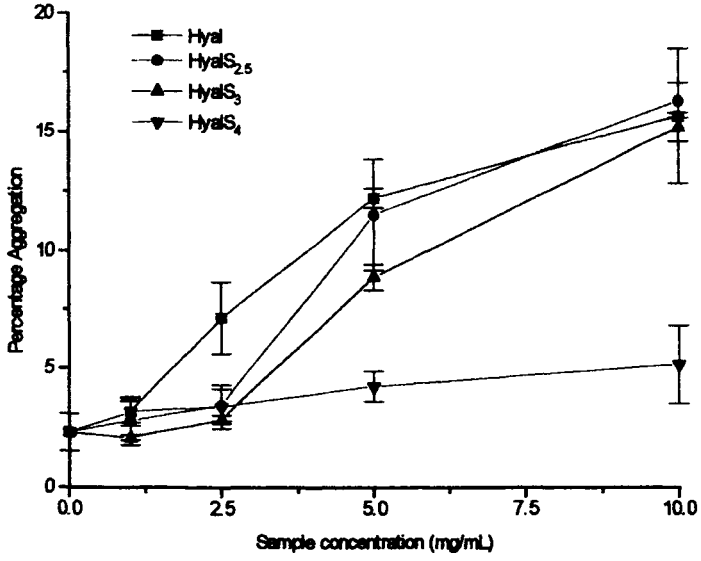
[a] 6.45×10^{-3} mg/ml; [b] the solution was diluted 1 : 5 with PBS 0.1 M.

factors on the observed prolongation of both TT and WBCT like, for instance, a non-specific adsorption of fibrinogen. Also the ability to inactivate thrombin, by both direct electrostatic interaction and HC II, increased when increasing the sulphation degree, as observed by the fibrinoformation time (Table 3). On the contrary, the $HyalS_x$ were not able to inhibit thrombin via AT III, as the same FT values were obtained with or without the cofactor.

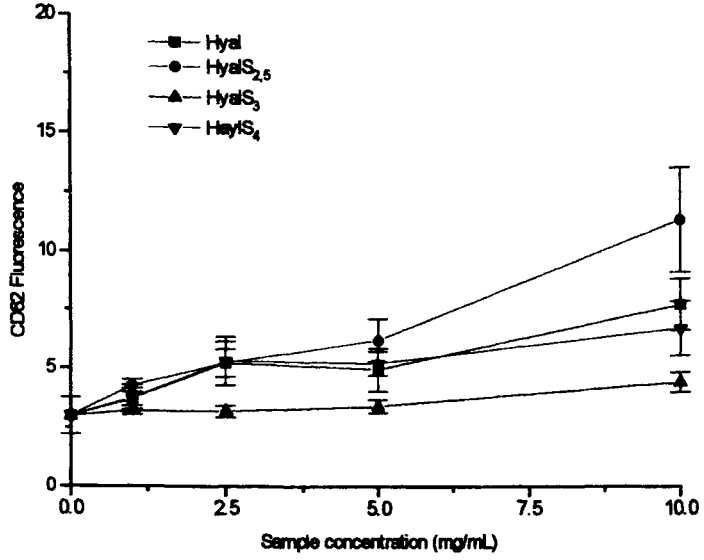
The Factor Xa inactivation was also studied. All $HyalS_x$ exhibited poor anti-thrombotic activity which modestly increased with increasing the sulphation degree.

Interaction of $HyalS_x$ with platelets

The ability of $HyalS_x$ to induce platelet aggregation was demostrated to be correlated to the polysaccharide degree of sulphation, as shown in Fig. 9(A). For hyaluronic acid the percentage of aggregation increased almost linearly with increasing concentration. $HyalS_{2.5}$ and $HyalS_3$ did not induce aggregation until a concentration of 2.5 mg/ml. Above this, platelet aggregation increased with increasing $HyalS_x$ concentration up to the same level as that observed with Hyal, at 10 mg/ml. $HyalS_4$ did not show any platelet aggregation, even at high concentrations. Considering that platelet aggregation generally occurs by an electrostatic interaction between the negatively charged platelet membranes and some positive residues on macromolecules [8], the different trends of both Hyal and $HyalS_x$ in causing platelet aggregation may be explained by the different chemical structures of the tested samples. Hyal presents as negative charges only COO^- groups which alone are not sufficient to electrostatically repulse the platelet membranes. In fact, the presence of a greater density of negative charges in $HyalS_{2.5}$ and $HyalS_3$ induces a stronger electrostatic

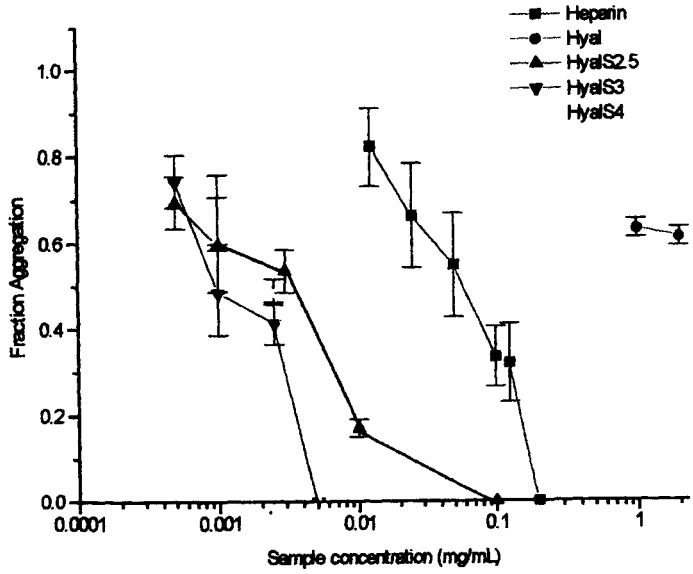


(A)



(B)

Fig. 9. (A) Platelet aggregation induced by different concentration of Hyal, $HyalS_{2.5}$, $HyalS_3$, and $HyalS_4$; (B) Platelet activation induced by different concentrations of Hyal, $HyalS_{2.5}$, $HyalS_3$, and $HyalS_4$.



(C)

Fig. 9. (C) In vitro inhibition of ristocetin-induced vWF-dependent platelet agglutination by unfractioneted Heparin, Hyal, $HyalS_{2.5}$, $HyalS_3$ and $HyalS_4$.

repultion, and aggregation can be observed only at concentrations of greater than 2.5 mg/ml. Furthermore, platelet aggregation is always inhibited by $HyalS_4$ carrying the highest number of negative charges.

Moreover, by analyzing the structure of the systematic series of synthesized macromolecules we can hypothesize that the –OH groups may be responsible for the interaction with the platelet membranes, as well as $-SO_3^-$ groups are responsible for their repulsion. The absence of –OH groups, such as in $HyalS_4$, excludes any change of interaction whilst the presence of four sulphate groups per disaccharide unit together with COO^- groups determines the largest platelet electrostatic repulsion.

The trend of platelet activation induced by contact with different concentrations of Hyal, $HyalS_{2.5}$, $HyalS_3$ and $HyalS_4$ was not dependent on the polysaccharide degree of sulphation as showed in Fig. 9(B). The activation was low for all the macromolecules tested. Some significant differences were observed among the different $HyalS_x$ at the highest concentration, with $HyalS_{2.5}$ inducing the highest response, higher than that of the simple Hyal which in turns was similar to $HyalS_4$. $HyalS_3$ produced a secretion almost indistinguishable from the control at all concentrations tested. This would indicate that the stimulation of α-granule released by $HyalS_x$ was via a different mechanism to that of platelet aggregation. Specifically, the controlling feature may not be due to an electrostatic interaction, but is likely connected with differences in the specific interaction of the macromolecule groups and receptors on the platelet surface.

The ability of $HyalS_x$ to inhibit von Willebrand Factor ristocetin-induced platelet agglutination depended on the presence of sulphate groups on the polysaccharide chain, roughly increased with increasing degree of sulfation and decreased with increasing $HyalS_x$ concentration, as shown in Fig. 9(C). Hyaluronic acid had negligible inhibitory activity, while $HyalS_{2.5}$ completely inhibited platelet agglutination at 0.1 mg/ml and both $HyalS_3$ and $HyalS_4$ induced the same effect even at lower concentration (0.01 mg/ml).

Bovine vWF, which aggregates platelets by the same mechanism as human vWF, but does not require ristocetin, was used to demonstrate the mechanism of action of $HyalS_x$. Adding bovine vWF to human platelets, they aggregate spontaneously but if bovine vWF and $HyalS_x$ are previously mixed, then the platelets do not aggregate. This demonstrates that the $HyalS_x$ binds directly to vWF hindering the binding to the platelet receptor GPIb, which permits the agglutination [9].

Consumption of CH50 units in presence of the studied compounds

The capacity of Hyal and $HyalS_{3.5}$ to activate the complement system was examined by incubating increasing amount of the polymers with serum for 1 h. The effects of the additions of the cross-linking quantities of Hyals or $HyalS_{3.5}$ to the serum, under the consumption of CH50 unities, were evaluated after 1 h of incubation. Figure 10 evidences that the presence of $\leq 1000\,\mu g$ Hyal per 1 ml of serum diluted at 1/4 in VBS^{2+} had no significant effect. Thus, this compound is not a complement activator under this condition. Besides, the presence of $Hyals_{3.5}$, comprised between 200 and 1000 μg causes a whole consumption of CH50 unities.

This compound, being a very charged polyanion, is able to complex with divalent ions necessary to the several steps of complement activation. We performed several dialyses on $HyalS_{3.5}$ solution against a high excess of VBS^{+2} and measured again the consumption of CH50 unities in the serun in the presence of the dialysed solution to check if this phenomenon was not the cause of the hemolysis absence of the erythrocytes used in the CH50 test. The results were identical to those mentioned above, which show the effect observed is not due to an important complex formation of the divalent ions.

Bidimentional immunoelectrophoresis of C3

The C3 cleavage was searched in the serum solution of $Hyals_{3.5}$ in bidimensional immunoelectrophoresis, after 1 h incubation at 37°C. The results evidence no significant difference between the tested solution and the serum. Thus, $HyalS_{3.5}$ is not a C3 activator.

The sodium salt of the tested hyaluronic acid (Hyal) does not consume CH50 unities in the tested concentration range. This result can be compared with that obtained with a dextran, having a molecular mass 70,000, used as plasma expander and which consumes small quantities of CH50 unities in the same concentration range

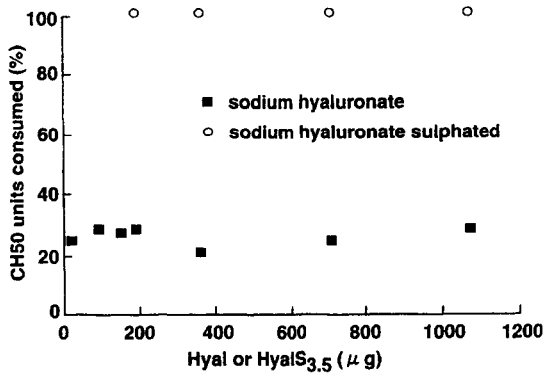


Fig. 10. Consumption of CH50 units in presence of Hyal and $HyalS_{3.5}$.

(about 40% per 1000 μg in the same system). Since the nanoparticles covered by this dextran are weak complement activators, it is likely that Hyal coated surfaces should not be strong complex activators. Nevertheless, as for dextran, this result depends on the conformation assumed by Hyal fixed on the surface and thus it had to be tested.

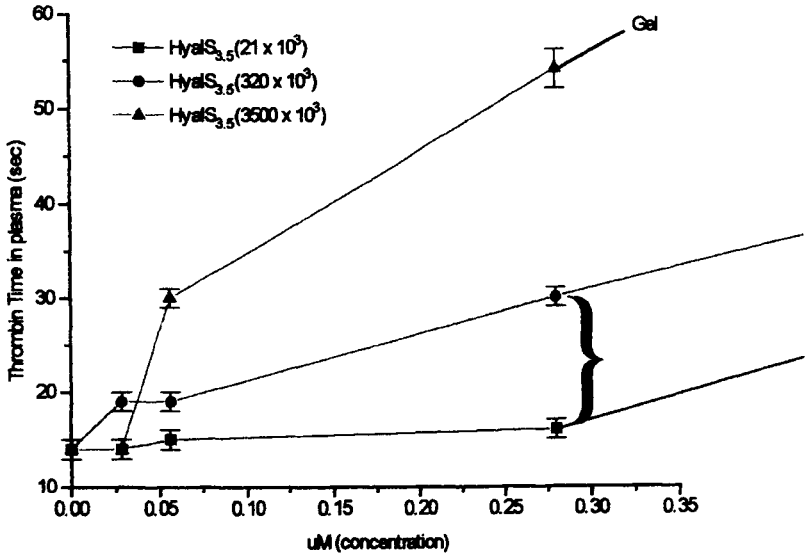
The results obtained with $HyalS_{3.5}$ are very encouraging. In fact, qualitatively speaking, this compound has the same outcomes of heparin in these systems. Heparin and $HyalS_{3.5}$ do not activate C3 and $HyalS_{3.5}$ is much more active than heparin to stop CH50 test. This effect is not due to a complex formation of divalent ions; it is likely that it is due to an inhibition of at least one of the processes involved in the lysis of erythrocytes by the complement. This should give interesting properties to surfaces coated with this compound in terms of complement activation.

Anticoagulant activity of $HyalS_{3.5}$ with different molecular weights

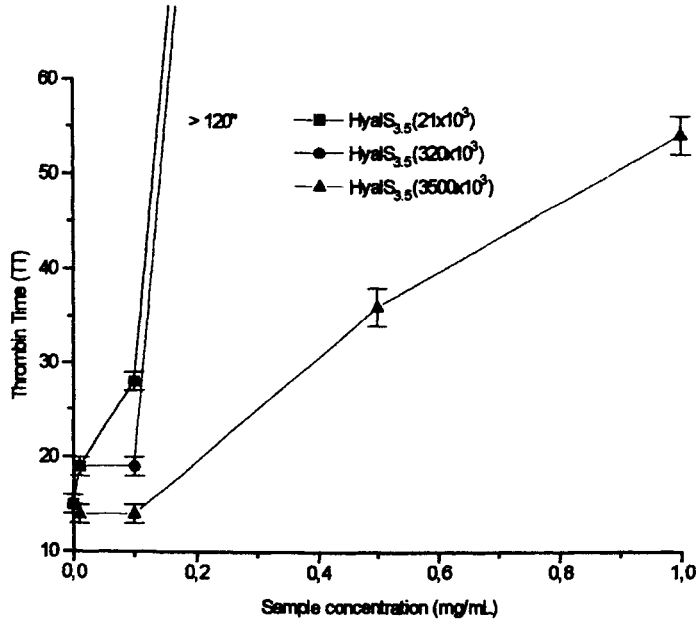
The ability of $HyalS_{3.5}$ to inhibit thrombin was demonstrated to increase with increasing molecular weight when the polysaccharides were compared in terms of molar concentrations (Fig. 11(A)). On the contrary, when the behaviour of the macromolecules was analyzed in terms of weight, the most reactive one was the $HyalS_{3.5}$ with the lowest MW (Fig. 11(B)).

These results underline the importance of analysing the properties of the macromolecules by choosing the best appropriate concentrations. In particular, the molar concentration allows us to distinguish the influence of the different length of the polymer, even if the number of repeating units is different according to the length of the molecular chain with the same molar concentration. Considering the concentrations in terms of mg/ml allows us to have the same number of disaccharide units even if the length of molecular chain is different.

Also the trend of Fibrino formation time (FT) of two different MW $HyalS_{3.5}$s (21 and 320×10^3) shown in Fig. 12(A) was similar to that observed in Fig. 10, but the FT of $HyalS_{3.5}$ with the highest MW remained constantly low before gelling. The same

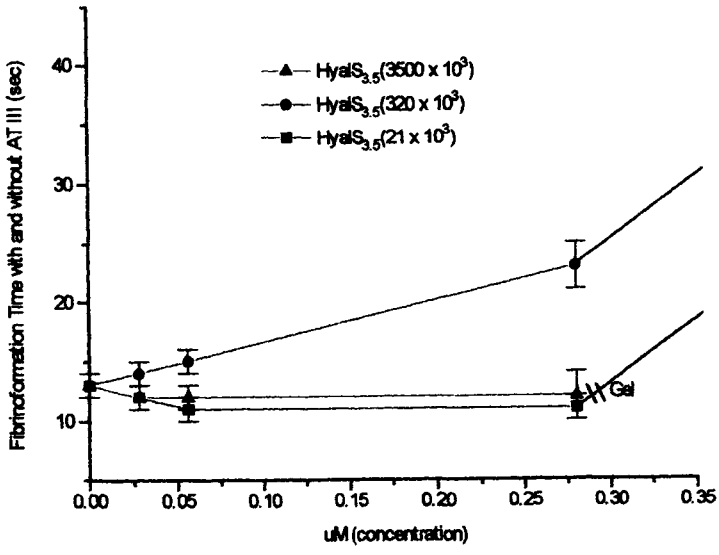


(A)

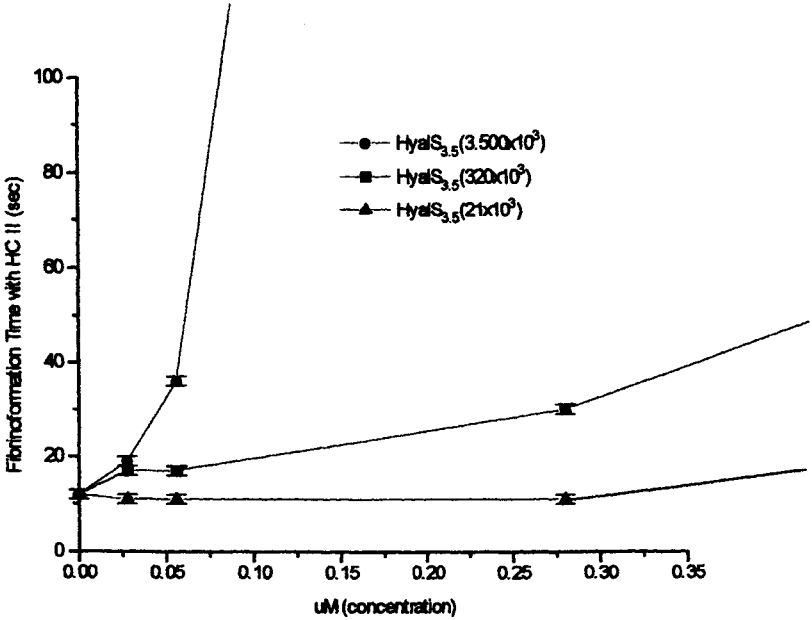


(B)

Fig. 11. (A) Thrombin time in plasma versus molar concentrations of different MW $HyalS_{3.5}$ (MW $\approx 21 \times 10^3$, 320×10^3, 3500×10^3). The molar concentrations were expressed in terms of the average real MW of the polymer chains and not as MW of the repeating unit; (B) Thrombin time in plasma versus mg/ml concentrations of different MW $HyalS_{3.5}$.



(A)



(B)

Fig. 12. (A) Thrombin inactivation (Fibrinoformation Time) with and without AT III versus molar concentrations of different MW $HyalS_{3.5}$ (MW $\approx 21 \times 10^3$, 320×10^3, 3500×10^3); (B) Thrombin inactivation (Fibrinoformation Time) in the presence of HC II versus different MW $HyalS_{3.5}$ (MW $\approx 21 \times 10^3$, 320×10^3, 3500×10^3) at high molar concentrations.

FT values were obtained in the presence of AT III showing that thrombin was inhibited only by direct aspecific interaction of the lower MWs $HyalS_{3.5}$ compounds with thrombin, whereas the highest MW $HyalS_{3.5}$ did not interact with thrombin.

In the presence of HC II too (Fig. 12(B), the ability of the compounds to accelerate the rate of thrombin inhibition increased with increasing molecular weight and even a jump in FT was recorded for the highest MW $HyalS_{3.5}$ at low concentrations, meaning that FT strongly depends on concentration.

Regarding FXa, all the samples slightly inactivated this serine-protease only in presence of AT III with a dependence on MW, but always precipitation was observed at relatively low concentrations. The concentration at which the solution becomes opalescent decreased as higher as the MW.

These results show the importance of MW in modulating the biological activity of sulphated hyaluronic acid derivatives. In fact, an increase in the MW was reflected by the concomitant increase in TT, but the influence of the three factors (AT III, thrombin, and HC II) was different at each MW. More specifically, the thrombin was inhibited via HC II and via direct aspecific interaction by the lower MW $HyalS_{3.5}$ compounds. On the contrary, the behaviour of the high MW $HyalS_{3.5}$ was mainly related to the electrostatic interaction with HC II. This macromolecule may assume a tight coil conformation in solution as demonstrated by the facility with which it gels. In this conformation it cannot freely interact with the other proteins, especially when the interaction is weak, as with thrombin. Since the interaction with HC II is stronger, the macromolecule can uncoil to form $HyalS_{3.5}$–HC II complex which inactivates thrombin. In this respect the behaviour of the three $HyalS_{3.5}$ compounds differs from that of heparin. Heparin affinity is stronger for AT than for HC II even if the heparin-HC II complex reactivity is greater towards thrombin.

The weak influence, if any, of AT III on these compounds can be explained by the fact that although the interaction between AT III and the polysaccharide is electrostatic in nature it can only occur for an optimal configuration of the macromolecule. Presumably $HyalS_{3.5}$ compounds cannot assume this particular structure, so this interaction contributes little to the mechanism of FXa inactivation.

Conclusions

It has been demonstrated that the sulphation reaction provides the hyaluronic acid macromolecules with a slight ability to interact with platelets in terms of both platelet aggregation and activation, also at relatively high concentration. Moreover, the introduction of sulphate groups along the Hyal polysaccharide chain makes the macromolecule resistant to the enzymatic digestion. In comparison with heparin, which is quickly degraded by heparinase when injected in the blood stream, $HyalS_x$ derivatives show a higher metabolic resistance.

All the mentioned biological characteristics render the sulphated hyaluronic acids suitable to be used in contact with blood either by alone, e.g. as heparin substitute, or connected with other blood substitutes.

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 27

Recent Developments and Future Perspectives for Preserved Platelets and Platelet Substitutes*

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Introduction

The development of frozen platelets and platelet substitutes is stimulated by the demand for the product and its very limited shelf life. Platelets are undergoing increasing use with an estimated 1.8 million transfusions in the US alone in one year. The shelf-life is 5 d at room temperature which assumes that platelet function and sterility will be maintained. Although the shelf life could be longer, there are currently no approved methods for ensuring sterility.

The function of platelets is to adhere to the subendothelium at the site of injury, undergo activation with expression of receptors such as glycoprotein IIb/IIIa and secretion of granular contents which then promote aggregation. The platelet is responsible for clot retraction, which occurs when fibrinogen binds to the activated GP IIb/IIIa receptor. In addition, the platelet provides phospholipids to enhance the clotting cascade.

The only quality criteria for platelets that are currently infused are that the count is $> 5.5 \times 10^{10}$ in single donor packs, $> 30 \times 10^{10}$ in bags obtained by platelet pheresis of a single donor, and the pH is > 6. Intact or relatively intact platelet function is assumed, although not measured; a one hour platelet count is used to assess response to the platelet infusion. The in vivo recovery of platelets that have been stored for 3 d or longer is approximately 70% of that found for fresh platelets. The decrease is due to the storage lesion, which is the end result of all the types of injuries that occur to platelets during storage.

Manufacturers and investigators in transfusion medicine are working in three aspects of platelet products to improve the supply. One focus is on sterilizing platelets to render them free from viral and bacterial infectivity. A second focus is on prolonging the shelf life through extended storage at 4°C, in the frozen state or in

*The opinions expressed herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of the Army or the Department of Defense.

Table 1

Platelet products or preservatives under evaluation

Platelet products or preservatives	Sources
Viral Inactivation	Cerus (Concord, CA)
Storage at 4°C or lower (Cryoprotectanats)	LifeCell Corp. (The Woodlands, Texas) COBE-BCT (Lakewood, CO)
DMSO	Naval Blood Research Laboratory, Boston
Freeze-dried	Centeon (Kankakee, IL)
Dried	Quadrant (Cambridge, England)
Platelet substitutes	IPM (Cypress Bioscience, Redmond, WA) Thrombospheres (Hemosphere, Irvine, CA)

the frozen and lyophilized state. A third area is in the development of parts of platelets or agents that mimic aspects of platelet function, and which will complement or enhance function of the platelets that are in the circulation. This chapter will describe the products being developed in these areas, along with in vitro assays and in vivo studies that are being done to assess function, as well as the clinical trials that have been initiated or are under consideration. A summary of some of the approaches to platelet storage as well as the companies involved in these efforts is listed in Table 1.

Sterilization of platelets for transfusion

Approximately 1 in 1500 units of random donor platelets is contaminated with bacteria; this number increases with prolonged storage [1–2]. Cerus has used a psoralen compound and long-wavelength ultraviolet radiation (UVA) to induce photochemical decontamination of platelet concentrates [3]. Platelets are treated with psoralen, which intercalates into the DNA and RNA. This is followed by brief exposure to UVA irradiation, which results in intra- and interstrand crosslinking. Since crosslinked DNA or RNA helices cannot undergo replication, viral growth in concentrates can be inhibited by this process. Cerus is currently studying a psoralen compound identified as S-59 (150 μM) and UVA (3 joules/cm^2), a low level of light that induces cross-linking in three minutes. The reaction takes place in platelets suspended in a balanced-salt solution that contains autologous plasma (35%) and additives which enhance buffering and platelet metabolism. The process utilizes special storage bags (Baxter Biotech) that transmit UVA. Cerus has also developed a device (S-59 reduction device) that can then remove the psoralen, which is known to be mutagenic.

This method inactivates viruses in lymphocytes and neutrophils as well as HIV in cell-free systems and viral surrogates for hepatitis B and hepatitis C. Gram-positive

and gram-negative bacteria (> 6 logs in colony-forming units/ml platelet concentrate) are also killed by this method [2–4]. After storage for seven days, the platelets still retain their function when tested in aggregation assays. As assessed in a rhesus monkey model, autologous psoralen-treated platelets have a recovery and lifespan that are similar to those of the untreated reference product.

A phase 1a crossover trial compared the post-transfusion recovery and lifespan of autologous five-day old S-59 treated platelets and untreated platelets in 23 volunteers. The platelets were treated with S-59 without the S-59 reduction device. This was followed by a phase 1b crossover trial, in which the safety of the S-59 treated platelets processed with the S-59 reduction device were compared with untreated platelets at full doses in 10 healthy volunteers. This study indicated that platelets treated with S-59 were well tolerated and safe. Sixteen volunteers were enrolled in a phase 2a trial which measured post-transfusion platelet recovery and lifespan of S-59 platelets that had been treated with the S-59 reduction device and then stored for five days. Treatment of the platelets with S-59 reduced the recovery and survival compared to untreated platelets but the results suggested acceptable performance (unpublished results). A phase 3 study is in the active planning stage.

Viral inactivation in platelet concentrates can also be accomplished with amino-methyltrimethyl psoralen (AMT) and UVA (320–400 nm) [5–7]. Treatment of platelet concentrates with AMT and UVA can be done in the presence of 0.35 mM rutin, a flavonoid that quenches reactive oxygen species. Photochemical treatment of the platelet concentrates by this methodology does not alter aggregation responses, morphology, or function as measured by a bleeding time in a thrombocytopenic rabbit model.

Prolonged storage of platelets

In order for platelets to change shape, the resting cytoskeleton must be altered. The first step in this change requires hydrolysis of membrane-bound polyphosphoinositides, which results in an increase in cytosolic calcium. A soluble protein known as gelsolin binds to calcium, which also increases its binding to filaments, thereby inducing their fragmentation. Through this process, discoid platelets are converted into spheres; 80% of the actin in the platelet is incorporated into filaments, which form the protrusions.

Cold-induced activation of platelets can be prevented by inhibiting the initial increase in calcium with the addition of 40 μM ethelyenebis *p* (oxyethylenernitrolo-tetraacetoxymethylester) (EGTA–AM) and by preventing filament elongation with the addition of cytochalasin B [8–9].

Platelets that are treated with EGTA-AM and cytochalasin B and then stored at 4°C for 21 d retain 80–90% of their initial function when tested in aggregation studies with thrombin as the agonist.

One group of investigators has used a murine model for studies of platelet function in vivo, since murine platelets do not have receptors for thrombin or for its peptide

mimic known as thrombin receptor activating peptide (TRAP), a peptide that activates the thrombin receptor of human platelets. In this model, human platelets which have undergone cold storage are labeled with a fluorescent probe and are then infused into mice, followed by administration of TRAP, which activates the human but not the murine platelets. The labeled human platelets can then be observed with microscopic techniques in small vessels after exposure to the TRAP. Human platelets stored in the presence of cytochalasin B and EGTA–AM at 4°C for 21 d retain excellent activity as assessed in this model.

Several laboratories have also used a process for freezing platelets for autologous transfusions. The degree of damage to the platelets induced by crypreservation with dimethyl sulfoxide (DMSO) may depend on the way in which the freezing process is performed. One group has shown that mean platelet volume is increased [10]. In addition, potassium, calcium, and lactate dehydrogenase are released from the platelets and they become activated.

Platelets are prepared by placing them in polyolefin bags to which DMSO is slowly added to achieve a final concentration of 5%. Platelets are then placed in the vapor phase of liquid nitrogen; freezing at a controlled rate does not seem to be an essential feature of preservation. Thawing is performed in a 37°C water bath, and then platelets are washed and resuspended in autologous plasma obtained at the time of platelet donation. The final washing step may not be essential.

Frozen platelets can still be hemostatically effective when stored for 4–5 years. They tend to give increments in the level of circulating platelets that are about half of what would be observed if the same dose of fresh platelets were infused into a non-immunized donor. In the oncology setting, autologous platelets that have been frozen are administered to alloimmunized patients who are receiving conventional or high-dose chemotherapy [11].

At the Naval Blood Research Laboratory in Boston, frozen platelets have been maintained at −80°C for up to three years [12]. The first step in the process is to store freshly-collected platelets for 24 h at 22°C to allow time for testing; the platelets are then frozen in 6% DMSO in a polyvinyl chloride plastic bag. Controlled-rate freezers and liquid nitrogen are not necessary. After thawing, the platelets are washed, thereby removing 95% of the DMSO. They can be stored up to six hours before infusion. Recovery in vivo is 70–75%.

The function of platelets has been assessed in patients who have undergone complex procedures such as a repeat coronary artery bypass procedure or cardiac valve replacement; non-surgical blood loss (defined as bleeding occurring after neutralization of heparin) was estimated after infusion of platelets. Patients were randomized in a prospective fashion to receive either liquid stored platelets (mean storage time, 3.4 d) or cryopreserved platelets (mean storage time, 289 d). Survival of liquid stored platelets was 39% compared to 23% of the cryopreserved platelets. The cryopreserved platelets significantly reduced blood loss over a 24-h transfusion period.

Two companies are also developing cryoprotectants that can eventually be

transfusable, thereby reducing the requirement for washing platelets before transfusion [13]. COBE BCT, Inc. (Lakewood, CO) is developing a transfusable cryprotectant which is comprised of glycerol and mannitol to influence the hydrogen bonding as the water freezes and a polymer (polyvinyl-pyrrolidone, PVP) to increase the glass transition temperature. The final formulation is 20% PVP (average molecular weight 2.5 k), 10% mannitol, 5% glycerol and a mixture of salts. PVP, which has a half-life of about four hours, is eliminated through the kidney. The process involves concentrating the platelets, removing the plasma, allowing platelets to "rest" for 60 min and then adding an equal volume of cryoprotectant with resuspension. Platelets are then placed in a mechanical freezer and stored at −80°C; controlled-rate freezing is not used. To prepare for use, the platelets are thawed at 37°C and then diluted with two parts saline and infused. The platelets prepared in this fashion have been altered as assessed by the hypotonic stress reaction and morphology score as well as increase in P-selectin expression; however, they appear to function well in the thrombocytopenic rabbit model.

LifeCell Corporation (The Woodlands, TX) has just obtained a patent for its product known as Thrombosol, which is comprised of a solution composed of adenosine, sodium nitroprusside, and amiloride, a sodium proton exchange inhibitor which blocks pH changes in platelets. DMSO can be added directly to achieve a 2% concentration (final). The platelets are stored in an aluminum cassette at −80°C without undergoing any controlled-rate freezing. After freezing for up to 17 d, the platelets were thawed at 37°C and then stored under blood-bank conditions at 22°C. By light microscopy approximately 66% were discoid in shape. With hypotonic shock, recovery was just under 60%. Expression of P-selectin was detected in 30% of platelets, a value to be expected in platelets stored at 22°C for two to three days. In a thrombocytopenic rabbit model, the platelet preparations were within the 95% confidence interval for normal bleeding time and platelet count. Future studies will be directed at further reduction in the concentration of DMSO to allow for direct post-thaw transfusion.

The Quadrant Holdings Co., Cambridge, UK is using trehalose, a disaccharide used for freeze-drying and stabilization of proteins and membranes, to preserve platelets [14]. However, platelets freeze dried with trehalose appear to be intact when in the dried state but undergo osmotic lysis when rehydrated. Additional studies are being performed with glucose, which fulfills the platelet requirement for sugar, and hydroxyethyl starch, which has a high glass transition temperature. Initial results with this mixture shows 40% responsiveness to thrombin after rehydration.

Rehydrated lyophilized platelets are also under commercial development by Centeon. With this procedure, washed platelets are exposed to 1.8% paraformaldehyde for 60 min, washed and then freeze-dried in the presence of 5% (w/v) albumin [15]. By light microscopy, reconstituted platelets have granularity and by flow cytometry fewer than 15% of the platelets are negative for surface glycoproteins GPIb or IIb/IIIa. If 20–30% of the platelets are positive for P-selectin, the preparation is considered to have undergone activation and will not be used in further studies

[16]. The Baumgartner model is used to assess adherence to subendothelium; fresh platelets are used for comparison. The reconstituted platelets appear capable of generating thromboxane B_2 to the same degree as fresh platelets and also enhance procoagulant activity when citrated plasma containing either fresh or reconstituted platelets is recalcified. Although the rehydrated platelets do not aggregate with ADP or collagen, they will do so in a 4 : 1 mixture of lyophilized:fresh platelets, suggesting that the lyophilized platelets could be involved in recruitment or cohesion of fresh platelets.

Studies in vivo include measurement of the ability of the platelets to shorten the bleeding time in thrombocytopenic rats. In this model, an antiplatelet antibody is administered at a concentration that induces thrombocytopenia of sufficient severity to prolong the toenail bleeding time to 15 min or greater. Infusion of rehydrated lyophilized human platelets corrects the abnormality. The platelet preparation also corrects the prolonged bleeding for at least two to three hours in aspirin-treated dogs undergoing cardiopulmonary bypass. In a thrombocytopenic rabbit model, the lyophilized platelets appear to be more hemostatic than would be predicted on the basis of platelet recovery.

The paraformaldehyde may also be virucidally (as well as bactericidally) effective. However, the immunogenicity and life span of platelets prepared in this manner have yet to be determined. It is also not known if subjects have naturally occurring antibodies that would recognize paraformaldehyde-treated platelets.

Platelet substitutes

Thromboerythrocytes are erythrocytes that are covalently coupled with RGD-containing peptides (approx. 10^6/cell). The length of the RGD peptide was designed to interact only with activated GPIIb/IIIa receptors, thus allowing thromboerythrocytes to react only with platelets that had become activated at the site of vascular injury. Their primary function in thrombocytopenia would be to augment platelet deposition at a site of injury [17–18]. Initial studies in vitro with current formulations showed that thromboerythrocytes could interact with platelets adhering to collagen under conditions of low shear rates of 50–100 s^{-1} but not at higher shear rates of 500 s^{-1}. The thromboerythrocytes did not significantly reduce the prolonged bleeding time in thrombocytopenic primates. However, reformulations of the product such as redesigning of cyclic peptides that have two to three logs higher affinity for GPIIb/IIIa than the linear peptides could alter these effects.

Thrombospheres (Hemosphere, Irvine, CA) which are microspheres (mean diameter of 1.2 micrometer) composed of crosslinked human albumin with human fibrinogen covalently bound to the surface are under development as adjuncts to platelets [19]. Thrombospheres shorten the bleeding time in a thrombocytopenic rabbit model. The effect is detectable within one hour post-administration and seems to last for at least 48 h. Moreover, thrombospheres co-aggregate with platelets in the

presence of a platelet agonist (but not in the absence of an agonist). It is possible that either or both of these effects occur through the enhancement of normal platelet activity.

However, the critical concentration of platelets needed for thrombospheres to fulfill this function is still being evaluated. Thrombospheres are not thrombogenic in the Wessler jugular vein stasis model, and they do not induce changes in circulating platelets or produce organ toxicity although this has been assessed only after single infusions.

Another substitute, an Infusible Platelet Membrane (IPM^{TM}) is being developed by Cypress, Inc. as a platelet substitute. A large fraction of the IPM^{TM} material is in the form of spherical vesicles which have a mean modal diameter of approximately 600 nm [20]. IPM^{TM} is prepared from 10–19 d old blood bank platelet units by first removing plasma, red cells and white cells through differential centrifugation [20]. The platelets are lysed by freezing and thawing and intracellular components removed by centrifugation. The product is heated at 60°C for 20 h to inactivate possible viral contaminants and then subjected to sonication and centrifugation. The preparation is formulated with sucrose and human albumin, lyophilized, and stored at 4°C. In lyophilized form the product is stable for more than 2 years at 4°C.

IPM^{TM} contains detectable GPIb but not GPIIb/IIIa and has procoagulant activity when tested as a source of phospholipid in a modified Russell viper venom assay. At a dose of 2 mg/kg IPM^{TM} shortened the prolonged bleeding time in thrombocytopenic rabbits with peak effect 4 h after infusion. IPM^{TM} was not thrombogenic in the Wessler jugular vein assay.

In phase 1 studies, IPM^{TM} was not antigenic and was well tolerated at a maximum dose of 6 mg/kg over 30–40 min. A phase 2 randomized, dose-ranging study evaluated the safety and efficacy of IPM^{TM} in patients (ages 18–70 years) who had moderate to active bleeding, and platelet counts less than 50×10^9/l. In the first cohort, 10 patients received IPM^{TM} (6 mg/kg) and two received random donor platelets.

Bleeding was defined as "resolved" if there was cessation without recurrence during the observation period or "improved" if bleeding decreased but was still present. In 7/10 patients treated with IPM^{TM} and in both patients receiving a platelet transfusion, bleeding decreased or stopped. 2/4 patients refractory to platelets had a response to IPM^{TM}, whereas 5/6 patients who were not refractory to platelets responded to IPM^{TM}. A history of refractoriness to platelet transfusions appeared to be a determinant in the initial response to IPM^{TM}. Furthermore, one refractory patient who did not respond to IPM did appear to have an increment in platelet count after receiving a platelet transfusion, suggesting that IPM may have at least transiently decreased the refractoriness to platelet transfusion.

In the second cohort, four patients, three of whom were refractory to platelets, received IPM^{TM} at a dose of 4 mg/kg. Two of the three refractory patients responded as did the one non-refractory patient. No patients experienced serious adverse events related to IPM^{TM} infusion with the exception of one patient who developed a shaking chill during one infusion. Results of coagulation studies, which included measure-

ment of fibrinogen-fibrin degradation products and platelet counts, were not altered by IPM™ infusion.

The advantages of IPM™ include minimization of viral and bacterial contamination as a result of heat treatment, potentially reduced immunogenicity due to reduction in class I HLA, prolonged shelf life (36 months) and absence of contaminating white cells and platelet components that could promote platelet transfusion reactions.

In vitro tests to assess function

Visual assessment of swirling is related to the refractile nature of platelets when they are in disc form; if they have undergone activation and have been transformed to spheres, they do not exhibit the phenomenon. Since discoid shape has correlated well with in vivo viability, assessment of swirling also has promise. In one investigation, the swirl phenomenon could be related to the pH of the concentrate; if swirling was satisfactory, then the pH was in the middle range [21]. Another useful assay is the evaluation of morphology by the Kunicki score, which reflects whether platelets are discoid, spherical, or dendritic. However, this test is quite subjective. A third method is to evaluate the response to hypotonic stress. An increase in light transmission occurs if platelets are suspended in water, since they undergo swelling. Functional platelets can actively pump water from the interior to the exterior, with a reduction in light transmission to a normal range (i.e. 100% recovery). Aggregation methods are unreliable, especially as performed with single agonists. Currently, experts in the area of platelet storage recommend measurements of platelet concentrate volume, platelet count and pH (ideal values between 6.8 and 7.4) after completion of storage [22]. Flow cytometry may be of use in evaluating platelet activation, and this methodology is now undergoing investigation [23]. However currently no single or even group of in vitro assays can substitute for in vivo studies in human recipients.

The Platelet Function Analyzer PFA 100™ System (Dade International, Inc., Miami, FL) measures platelet adhesion in vitro by determining the rate of blood flow or time to filter occlusion. It is usually performed by passing anticoagulated whole blood or platelet-rich plasma through a filter under a pressure gradient. Instruments utilized for the global measurement of hemostasis are the Thromboelastograph® Coagulation Analyzer Haemoscope, Morton Grove, IL and the Sono Clot ¥® (Sienco, Inc., Morrison, CO), both of which can measure the clotting time of nonanticoagulated blood. Platelet activity is responsible for the increase in the signal that develops with the clotting of whole blood. The Clot Signature Analyzer (CSA®, Xylum Corporation, Scarsdale, New York) measures the time required for closure of a puncture site in an artificial vessel through which the test sample (whole blood) is flowing at a constant pressure. The correlation of these in vitro techniques with clinically meaningful endpoints is still under investigation.

In spite of aggregatory functions being reduced in platelets that had undergone 5-d storage, and spontaneous P-selectin expression being increased, platelets still demonstrate excellent contractile function as measured with the Thromboelastograph® Coagulation Analyzer (TEG) [24–25]. The specificity of measurement of platelet function can be increased by measuring the signal or mean amplitude in both platelet-rich plasma and platelet-poor plasma after the addition of thrombin and calcium. The difference between platelet-rich plasma and platelet-poor plasma is considered as a measurement of platelet function.

The Baumgartner perfusion chamber [26] could be used to study platelets in vitro under conditions of flow. This system assesses elements of the blood, such as von Willebrand factor, essential to platelet function; furthermore, shear rates could be adjusted to reflect conditions in vivo. The Baumgartner apparatus is made by placing a de-endothelialized everted rabbit aorta on the inside rod of an annular flow chamber; when blood flows through the chamber, platelets are in contact with the de-endothelialized surface. At the end of the experiment, the surface is scanned for the platelets deposited. Results can be expressed as a percentage of surface that is covered by adherent platelets in contact with, or spread over a surface area or that have formed platelet thrombi.

In vitro or on vivo systems that assess platelet function under different shear rates could also be developed to assess function of platelet products. Shear rates range from $50\,s^{-1}$ in the vena cava to as high as $15{,}000\,s^{-1}$ in atherosclerotic stenotic vessels. At low shear rates, interaction of platelets with the vessel wall is diffusion controlled, and at high shear rates, the interaction is due to actual reactivity of platelets with the surface.

Erythrocytes are important in concentrating platelets near the vessel wall by rheologic properties and rotational movement; they may also increase platelet reactivity by metabolic means. At high shear rates (i.e. $2{,}600\,s^{-1}$) von Willebrand factor and not fibrinogen is important for interacting with GPIIb/IIIa.

The use of animal models to assess platelet products

In a canine model, autologous platelets have been biotinylated ex vivo and then reinfused [27–28]. Blood samples were then drawn at regular intervals and the platelets were then tagged with phycoerythrin-labeled strepavidin and analyzed by flow cytometry. In a baboon model, Michelson et al. [29] used a three-color whole blood flow cytometric analysis to distinguish between infused labeled platelets and noninfused platelets in the same sample. Baboon platelets were labeled with PKH2, a fluorescent lipophilic dye that binds to the platelet membrane. Using a three color method they were able to simultaneously identify platelets by the binding of R-phycoerythrin-conjugated GPIIb-IIIa-specific mAB 7E3, distinguish the infused platelets by their PKH2 fluorescence, and determine the platelet surface expression of P-selection on individual platelets by using biotinylated mAbs S12 or G1. Their study showed that although platelets lose their P-selectin to the plasma pool, they

continue to circulate and function like degranulated platelets that do not localize at a thrombus. However, the above methods do not avoid the problem of damaging modifications that may occur in preparing the platelets before infusion into the animal.

Aging of platelets and function can be measured by removing biotinylated platelets serially, inducing activation with thrombin, and measuring P-selectin expression [30–31]. Platelet function can also be measured in a canine model by creating an arteriovenous shunt into which a silastic tubing containing a cotton thread as a thrombotic surface is inserted. At the end of the study, the string is removed, and platelets are released by digestion with chymotrypsin.

In a thrombocytopenic rabbit model, platelet counts are reduced with a sublethal dose of radiation from a Cesium source at day −10 followed by an injection of sheep anti-rabbit platelet antibody at day −1. This results in a platelet count $< 10 \times 10^9$/l; platelet counts then increase beginning at day 10 after radiation. If platelets from humans are to be studied, the animals are first injected with ethylpalmitate on the day of platelet infusion to block the reticuloendothelial system, thereby preventing rapid clearance of human platelets. A bleeding time is measured in an ear warmed to 37°C followed by a full thickness incision 9 mm in length. The ear is placed again in a stirred 37°C water bath (two liter beaker) so cessation of bleeding is easily noted. The bleeding time is measured for 15 min. The volume of blood loss can also be quantitated with chromium-labeled red cells. In this model young platelets are more hemostatically effective than older platelets and also have a longer survival [31]. When infused into rabbits pretreated with ethylpalmitate, human platelets have a survival half-life of approximately four hours, and this is not affected by splenectomy. Human platelets that have been stored at 4°C for 24 h are also hemostatically effective in this model. However, hemostatic function and in-vivo survival are not necessarily correlated. For example, in human volunteers, autologous platelets stored at pH 6.4 for 10 d at 4°C also had hemostatic activity; however, circulation time was reduced.

Human platelets frozen in DMSO, stored for one to two months and then thawed, washed and infused into thrombocytopenic rabbits had hemostatic activity. Platelets treated with psoralen and UVA also had good hemostatic function in this model [32]; however, lyophilized platelets had reduced hemostatic function when compared to the same concentration of liquid-stored platelets.

Several models have also been used to demonstrate thrombogenicity of such products as Factor Xa and liposomes [33–34] consisting of phosphatidylcholine: phosphatidylserine (80 : 20). This preparation was toxic in dogs, inducing a decrease in factors V and VIII as well as in the platelet count and hematocrit. When tested in fawn-hooded rats with a storage pool disease, the preparation shortened the bleeding time of a tail laceration without affecting the actual volume of blood lost. Studies in vitro showed that this preparation prevented the inhibition of factor Va by activated protein C. Furthermore, in a baboon model, the toxic effect of factor Xa-phospholipids was greatly increased when protein C activation was blocked by infusion of a monoclonal antibody. Although these studies were performed with factor

Xa/liposomes, the models could potentially be used to investigate the thrombogenicity of platelet preparations.

Platelet substitutes: general considerations

Ideally, platelet products that are developed for infusion should undergo adhesion, release agonists or adhesive glycoproteins from alpha granules that can promote tissue repair, and release plasminogen activator inhibitor-1 to promote clot stability. The main reason for developing platelet products is to treat hemorrhage due to thrombocytopenia. For the present time, post transfusion platelet counts are being used as markers for efficacy whenever possible. The bleeding time is not useful as currently performed since hemostasis may be corrected yet the bleeding time remain prolonged.

Platelet products could lead to several potential adverse biologic activities. One of these, potential thrombogenicity, could be due to platelet particles which could promote procoagulant activity. Platelet particles have been found in fresh frozen plasma; the particles are present in even higher concentration in the cryoprecipitate. An additional potential but extremely unlikely mechanism for toxicity is that nonfunctional platelets could actually interfere with normal platelet function in thrombocytopenic patients.

Due to the heterogeneity and complexity of the clinical setting in which platelets are used candidate platelet products will be difficult to evaluate since the efficacy of current products, which are FDA licensed single-donor platelets or random donor-platelets, is not well defined.

One approach is to demonstrate with in vitro assays that the platelets had not been damaged. A device that quantitates light scattering has been developed to assess platelet shape by measuring optical changes in stored platelets [35]. The result is expressed as a light scattering index, and can indicate the percent of platelets in a bag that are discoid, as well as the platelet count. However, the value of this test, as is the case for any in vitro test in use, has not been correlated with function in vivo. A second approach is to show the ability of platelets to circulate in vivo, using autologous platelets subjected to the treatment under evaluation; and the third is demonstration of clinical hemostasis.

Product safety is usually determined in a phase 1 study with normal volunteers or patients. Efficacy or activity of the product is studied, with safety still under consideration, in phase 2 trials. Phase 3 trials are the controlled trials in which the safety and efficacy of the product are determined in a definitive manner although a product may undergo phase 3 testing and then undergo additional safety studies. Furthermore, preclinical studies may continue even though the product is in phase 2 testing. The phase 3 trial is designed to demonstrate efficacy, which is clinical benefit (defined as improved patient survival, or prevention or decreased progression of disease). However, federal regulations will allow the use of a surrogate endpoint if

achievement of clinical benefit cannot be tested and if the surrogate endpoint is appropriately validated.

Platelets are being frozen and used in patients; efforts are also underway to develop freeze-dried platelets. The technology to produce may different kinds of products is in place. The products may range from membranes to reconstituted freeze-dried platelets. The major goals are now to develop ways to assess each of these products with studies in vitro and in vivo. The methods will not be universally applicable to all products; and products may vary in their indications for use. Finally, markers of activity that are clinically useful are still being developed; the success of these efforts will impact on patient care.

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Blood Substitutes — Present and Future Perspectives
E. Tsuchida (Editor)

CHAPTER 28

Development and Clinical Implications of Platelet Substitutes

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Introduction

Platelet transfusion plays an important role as a supportive therapy in the treatment of cancer or hematologic malignancies and during the surgical procedures. Recently, usage of platelet concentrates increased annually by approximately 10%, and it would further expand with the development of medical treatment in coming 21st century. However, there are two important issues to be solved in platelet transfusion [1–3]. One is the increase of demand and the short-term storage of platelet concentrates. The other is the risk of platelet transfusion such as viral infections. In order to solve these problems, some academic societies as centers of excellence are trying to make guidelines for platelet transfusion to decrease the number of unnecessary transfusion. Different from red cell transfusion, autologous platelet transfusion is rarely indicated. In this sense, the development of artificial platelets or platelet substitutes and the exploration of their clinical applications are reasonable ways in the aim of medical treatment of the coming century. Platelets could be stored for several days at room temperature, over that period they lose their hemostatic functions dramatically. In Japan, storage periods for platelet concentrates have been decided to be 72 h after donation, during the periods quick inspection and supply of the adequate type of preparations are necessary. Generally speaking Japan, which has well-equipped national blood programs with a large number of volunteers who are willing to donate blood, is not facing serious problems in supplying platelet concentrates. However, this is not always true in depopulated areas and in cases of emergency. Therefore, the development of artificial platelets or platelet substitutes which are available in any situations would contribute to the effective blood programs against disasters; furthermore, it brings a large impact to completely remove the risk of infection due to transfusion.

Platelet functions necessary to be replaced

Platelets have diverse functions such as adhesion, aggregation, clot retraction, procoagulant activity, etc. It seems difficult to develop platelet substitutes having

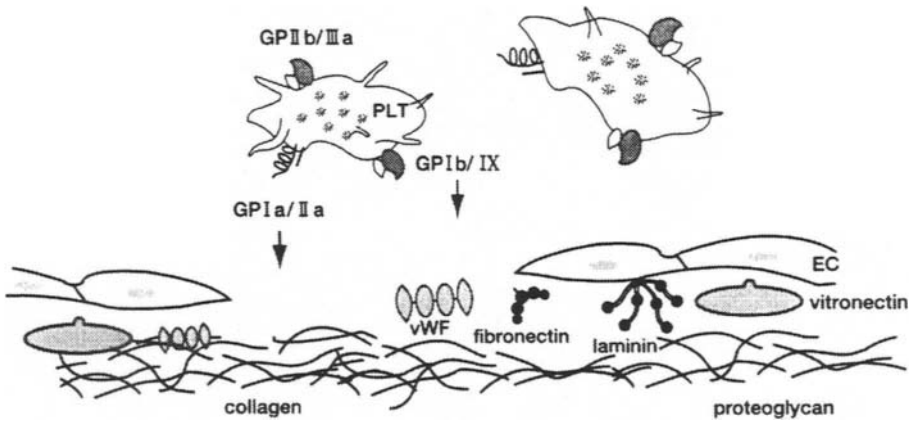

Fig. 1. Major adhesive ligands in subendothelial tissues and their platelet receptors. Glycoprotein (GP) Ia/IIa and GPIb/IX are receptors for collagen and von Willebrand factor (vWF), respectively. GPIIb/IIIa binds to several adhesive proteins including fibrinogen, vWF and fibronectin.

all these functions. The platelet substitutes for the time being is considered to be enough to have the limited functions of adhesion and aggregation appearing at the bleeding site in order to plug up a hole of blood vessels, and to help the functions of remained normal platelets.

The basic and important functions of platelets are adhesion and aggregation for primary hemostasis. This can be easily understood from the observations that congenital platelet membrane defects such as Bernard-Soulier syndrome or Glanzmann's thrombasthenia, which are deficient in platelet adhesion or aggregation show severe bleeding tendency. Therefore, the simplest type of artificial platelets is a particle, of which surface membrane proteins working as receptors are immobilized onto. Points to consider are (1) what kind of particle would be used as a carrier; (2) what kind of proteins would be immobilized in order to exhibit such platelet functions. Requirements of the former point are appropriate size, no sign as a foreign body, and a good carrier to be able to immobilize enough amount of proteins. The latter would be GPIb/IX complex; a receptor of von Willebrand factor (vWF) which is important for adhesion, GPIa/IIa complex; a receptor of collagen, or GPIIb/IIIa complex; a receptor for fibrinogen or vWF, which is necessary for aggregation (Fig. 1).

Current status of development of artificial platelets

Development of artificial platelets or platelets substitutes is still in the premature stage. In 1996, US Army Combat Casualty Care Research Program, Naval Medical Research and Department Command held the first meeting entitled "Frozen Platelets and Platelets Substitutes" cosponsored by FDA, AHA, etc., at Washington DC. In this

meeting, the necessity of platelet substitutes and their evaluation methods were firstly discussed and then present status of up-to-date research works were reviewed. Figure 2 is a list of the materials which have the possibility of being developed as platelet substitutes. Agam et al used normal human red blood cells as a carrier of fibrinogen [4]. The modified red blood cells are incorporated into aggregates when mixed with normal platelets and shorten the bleeding time after being infused into rats with thrombocytopenia. The modified red blood cell has the same structure, osmotic response, hemoglobin concentration and acetylcholine esterase activity as normal red cells.

Coller et al. produced modified red blood cells, so called thromboerythrocyte [5]. The modified erythrocytes have synthetic peptide chains extending from its surface. The peptide has a sequence of Ac–CGGRGDF–NH_2; especially the RGD sequence is the part of amino acid sequence of fibrinogen and relates to its role in binding to GPIIb/IIIa. It was shown that the modified erythrocytes participated in aggregation through specific reaction with platelets having activated GPIIb/IIIa on their surface. The thromboerythrocytes were reported to induce aggregation in the presence of ADP. This was clarified to be specific aggregation via GPIIb/IIIa because aggregation

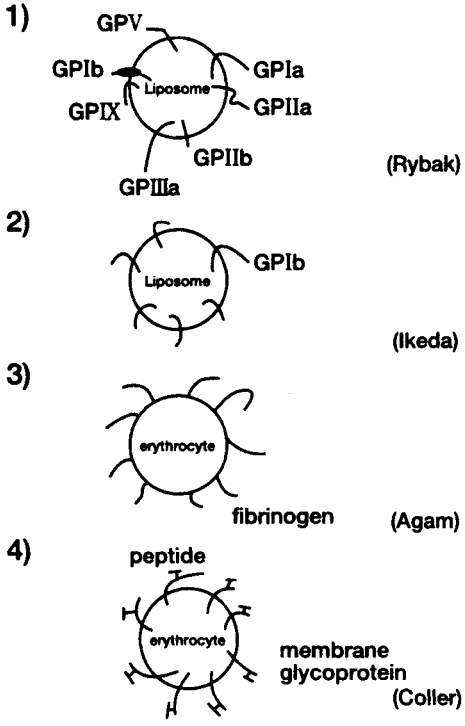

Fig. 2. Substances developed for potential use for platelet substitutes. (1) Plateletsome; (2) rGPIb α-liposome; (3) Fibrinogen-RBC; (4) Thromboerythrocyte.

was inhibited by anti-GPIIb/IIIa monoclonal antibody, 10E5. Furthermore, because thromboerythrocytes are adhesive against plateletes adhered on the collagen surface, it has a possibility to reinforce the platelet plugs at the injured vessels of thrombocytopenic patients. There was no hemolysis during preparation and high resistance against osmotic change. In clinical settings autologous red blood cells from a patient are manipulated outside of the body and then reinfused safely to the patient to control the bleeding tendency.

Recently, Rybak et al. developed liposome-type platelet substitutes, plateletsomes [6]. Liposome, phospholipid vesicle, is used as a carrier to which more than 15 kinds of platelet membrane proteins such as GPIb, GPIIb/IIIa, GPIV etc., were immobilized after dissolving them with deoxycholate from native platelets. Infusion of the plateletsomes into rats with thrombocytopenia induced by irradiation or rats with platelet function abnormality like storage pool deficiency caused significant reduction of blood loss from the cut tail of rats. Because no suppression or enhancement of in vivo platelet functions and no severe side effects were recognized, it is noteworthy as one candidate of platelet substitutes.

On the other hand, Cypress in US developed a powder product, Infusible Platelet Membrane (IPM), from human platelets [7,8]. At present, this is named as Cyplex^{TM}. The products were prepared from fractions of outdated platelets by freeze-thawing cycles, heat and drying processes. It was reported that they could be stored about 36 months at 4°C. Although there is no report on the detailed description about constitution, main components were reported to be phospholipids and glycoproteins, and their size was about one tenth of normal platelets. In products, the presence of GPIb was confirmed with ELISA, but the other glycoproteins such as GPIIb/IIIa were not detected. This seemed to be due to the loss of antigenic determinants during the preparation procedure. They have procoagulant activity of 170–330 U/mg protein. Activities of the other coagulation factors such V, VIII, IX, and X etc., and HLA antigen were not observed. After the injection (2 mg/kg) of Cyplex^{TM} to rabbits with thrombocytopenia induced by busulfan injection, the shortening of bleeding time was recognized. This effect continued for about 6 or 7 h, followed by the return of bleeding time to an initial value. Using several kinds of animals, no significant toxicities were observed by biochemical, histopathological tests. Induction of clot formation was not recognized, and endotoxin-induced DIC was not deteriorated. In experiments examining virus decontamination by heat treatment, the decreases in about 6 log for SV40, HIV, or CMV viruses were confirmed. The Phase I and II data are summarized in Fig. 3. The advantages of Cyplex^{TM} are as follows: (1) The low risk of infection because of heat treatment; (2) low antigenicity due to the absence of HLA (class II); (3) long-term storage (36 months); (4) no febride reaction due to the absence of leucocyte; and (5) possible efficacy in refractory patients against platelet transfusion. The drawback was pointed out that the mechanism of action was unclear. Probably, the adhesion of particles having GPIb antigen to the bleeding site of subendothelial tissues, facilitate the platelet plug formation by remained normal platelets as a result of recruitment of plasma vWF

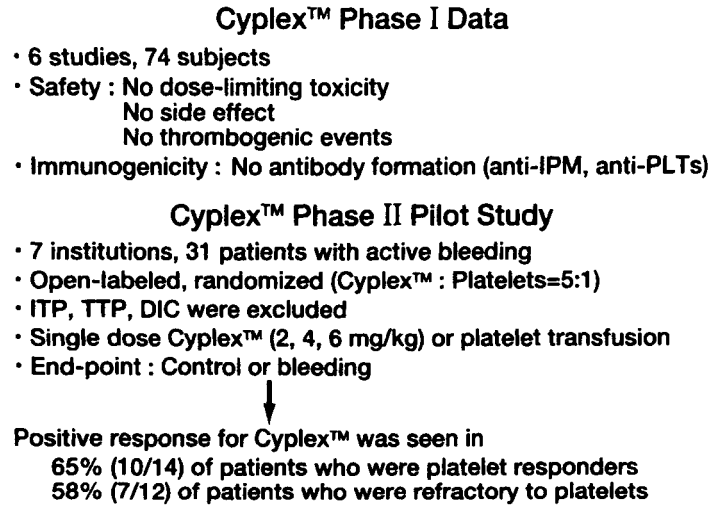

Fig. 3. Clinical trials of Cyplex™. Phase II pilot study has been completed by the end of 1997. Phase II will be continued in 1998.

onto the surface of the particles. Or there is a possibility that their activity of clot retraction would be responsible for an effect on hemostasis.

rGPIbα-liposome as a novel platelet substitute

Platelet glycoprotein (GP) Ib/IX complex is a receptor for von Willebrand factor (vWF), which plays a crucial role in primary hemostasis. We have previously expressed in CHO cell a domain of GPIbα (residues 1–302) retaining a vWF-binding site and have purified with affinity column chromatography [9]. Based upon a hypothesis that liposomes capable of participating in the hemostatic process may become possible platelet substitutes, we have attempted to construct liposomes carrying vWF-binding domain of platelet GPIbα. First, GPIbα was conjugated with *N*-glutaryl-phosphatidyl ethanolamine (NGPE) to synthesize GPIbα-lipid for the incorporation of this recombinant fragment (GPIbα) into the surface of liposome [10]. rGPIbα-lipid was introduced onto the surface of the liposome, which was consisted of egg-yolk lechitin/cholesterol/phosphatidylglycerol (10 : 5 : 2 by mol.). A final lipid concentration was about 1.4 mg/ml, while GPIbα protein concentration 1 mg/ml. The average diameter of lyposome was approximately 330 nm. rGPIbα on the liposome surface was detectable by flow cytometry using FITC-labeled anti-GPIbα monoclonal antibody, GUR20-5. To study the agglutination of the rGPIbα-liposomes in the presence of vWF as a ligand of GPIbα, vWF was added to an aqueous solution of rGPIbα-liposomes at a final concentration of 50 μg/ml, and agglutination of rGPIbα-liposomes was monitored by an aggregometer PA-100

(Kowa, Japan), that measures changes in light scattering. It was confirmed that the agglutination of rGPIbα-liposomes was demonstrated in the presence of vWF only when 1 mg/ml ristocetin was added to the system. Furthermore, such agglutination was completely blocked in the presence of 50 μg/ml of anti-vWF monoclonal antibody, NMC-4 (a generous gift by Dr A. Yoshioka, Nara Prefectural Medical School), [11] suggesting the specific agglutination. We also confirmed this morphologically using the rhodamine-labelled rGPIbα-liposomes under the fluorescent microscopy. Rhodamine-labeled rGPIbα-liposomes supplemented with FITC-labeled vWF was agglutinated by ristocetin. Rhodamine was detected in all conditions, but FITC was detected only under conditions of which both vWF and ristocetin were added. This agglutination was also inhibited by GUR20-5 and NMC-4, indicating the specific binding between rGPIbα and vWF. Next, we studied to clarify whether heterologous aggregation, i.e. attachment of liposomes to platelets, would occur or not. ^{3}H-labeled rGPIbα-liposomes were first mixed with platelet rich plasma, then ristocetin was added to the mixture to induce platelet aggregation. After centrifugation, pellets were counted for radioactivity. Involvement of rGPIbα-liposomes to platelet aggregates was evident. This was inhibited by monoclonal anti-vWF antibody NMC-4, suggesting that the reaction is specific. The specific inclusion of rGPIbα-liposomes was clearly demonstrated dose-dependently.

The findings that rGPIbα worked as a receptor on the surface of the liposome and required ristocetin to react with soluble vWF strongly suggest the similar behaviour to native platelets. In other words, these preparations would not cause aggregation in blood stream when infused and could adhere to structurally modified vWF, e.g. vWF immobilized on the subendothelial tissues. Furthermore, rGPIbα-liposomes were incorporated into platelet aggregates and enhanced platelet aggregation. It could be speculated that rGPIbα-liposomes may bind vWF and accumulate on exposed subendothelial tissue in vivo, serving as platelet substitutes supporting hemostasis in thrombocytopenic individuals.

Perspectives of artificial platelets

Ideal properties of artificial platelets

In clinical applications of platelet substitutes, important points are which platelet functions they are expected to substitute and to what degree they are expected to substitute. So far various artificial organs or tissues have been developed; however, there seems to be several factors which would determine their possibility. Namely, they are the number of functions of objective organs or tissues and their degree of complexity, constant or occasional appearance of the functions (the control of function), partial or complete substitution of the functions, temporal or permanent substitution etc. Usually, artificial organs can be divided into a hybrid type (coexistence of natural and artificial parts) and a totally artificial type. In the case of platelet substitutes, improvement of a cell fusion technique or genetic engineering,

and combination of natural tissues or cells with artificial products, materialization of platelet substitutes having the functions closer to natural platelets would be developed. It is considered to be impossible to realize the hemostatic function of platelets with artificial products only. A hybrid type should be more realistic in order to have the complex modulation function of normal platelets such as effective plug formation at the bleeding site only and dissolution of the plugs after restoration.

No risk of infection by transfusion is one of the primary requisites. Beside this, long-term preservation would be also extremely important in depopulated areas. They should be easy to use in a case of emergency. Their efficiency should be maintained after repeated infusions not to make antibody and not to cause serious allergic reactions. More important requisite is that infused platelet substitutes should not function in blood stream to make plugs. This is specific to platelet substitutes and different from artificial red cells or the other transplanted organs. In other words, a major promise is to switch-on their functions at the wounded site of the vessel only. Membrane glycoproteins are receptors, which are important for platelets to play their hemostatic roles. Usually, they are inactive states, and become active at the wounded sites where subendothelial tissues are exposed to make platelet plugs. Ideal platelet substitutes should have this modulation mechanism, and the plugs formed by the platelet substitutes should be dissolved and absorbed in the body after completion of hemostasis and restoration of the tissues. Furthermore, the platelet substitutes should not suppress the normal platelet functions, and the production of normal platelets should not be inhibited by a feedback mechanism. The platelet functions are complex such as adhesion, aggregation, secretion and procoagulant activity, and they should occur whenever and wherever they are requested to work. The artificial platelets having all these functions are too difficult to be developed. However, clinical purpose would be satisfied if one could give some hemostatic abilities to artificial particles, which could facilitate the physiological functions of remaining platelets or coagulation factors.

Research and development of platelets substitutes in Japan

Research on artificial platelets or platelet substitutes has just started, and, in order to produce clinically applicable artificial platelets or platelet substitutes, it is necessary to accumulate basic researches step by step. In 1997, a big research group on the development of artificial platelets was awarded Health Sciences Research Grants (Artificial Blood Project) from the Ministry of Health and Welfare, Japan. The characteristic features of this research group are first to establish basic theories about fluid mechanics and hemostatic thrombus formations to make ideal artificial platelets or platelet substitutes and appropriate evaluation methods for ideal clinical applications of the products. The main objective in 1997 was to make particles which accumulate specifically at the injured sites of the blood vessel. The targets for adhesion are collagen within subendothelial tissues or vWF, and the liposomes having the ability to bind them have been prepared. rGPIbα as a receptor of vWF

was prepared in large quantities by a recombinant technique. The rGPIbα-liposomes showed an adhesive ability and coaggregation with human normal platelets. The in vivo evaluation is planning. A system for continuous measurement of human platelet adhesion to collagen under the flow conditions is established and the importance of platelet adhesion through the $\alpha_2\beta_1$ integrin of platelet membrane was clarified. $\alpha_2\beta_1$ integrin was produced as a water-soluble protein complex by a recombinant technique, and its introduction to the liposome to prepare $\alpha_2\beta_1$-liposome is now being planned to evaluate its function as well as rGPIbα-liposome.

On the other hand, there are some problems such as the short half-life in blood circulation, or phagocytosis by monocytes in liposome-using systems. The development of the supplementing method and the experiments using polymers made of proteins as carriers other than liposomes are planning. Different from artificial red cells, the development of artificial platelets or platelet substitutes has just started. However, after fundamental data to create artificial platelets having hemostatic activity are established and the entire figure of the prototype artificial platelets is clarified through the animal experiment, the further improvement is needed in order to afford the advanced platelet substitutes in its shape and physicochemical properties. Toward this purpose, it is important to arrange the knowledge of human platelet behavior under the flow conditions based on the fluid mechanics and model experiments. The other important approaches are to understand the molecular mechanism of platelet adhesion for the effective accumulation to the wounded site of the vessel and reinforcement of fragile vascular wall in thrombocytopenic states. The research of matrix protein existing on the vascular wall or collagen at the wound site of vessels and corresponding receptor of platelet membrane protein should be proceeded simultaneously.

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Blood Substitutes — Present and Future Perspectives
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CHAPTER 29

The Impact of Blood Substitutes on the Blood Program

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Introduction

The major tasks of the national blood program are the establishment of transfusion safety and the self-sufficiency of blood products. The elimination of untoward transfusion-associated side effects, especially allosensitization and transfusion-transmitted infectious diseases are important goal in transfusion medicine. The accomplishment of domestic self-sufficiency in blood and plasma component products are also strongly requested by WHO, since Japan has been consuming approximately 30% of worldwide used albumin. Very recently, coagulation factor VIII has become available in a recombinant form. In addition, recombinant human albumin will be artificially produced within several years. Artificial oxygen carriers are also expected to be applicable as substitutes for red blood cell transfusion. These substitutes will greatly modify the ongoing transfusion medicine by reducing the chance of allogeneic transfusion. As well, critically ill patients suffering from massive hemorrhage at an emergency condition will be rescued by use of artificial oxygen carriers. The potentials and the roles of these blood substitutes in transfusion medicine should be addressed.

Demand and supply of blood products in Japan

Whole blood and blood component products

Japan has already established self-sufficiency in whole blood and blood components for transfusion [1]. The changes in the number of blood donors are shown in Fig. 1. In 1986, 400-ml whole blood donation and apheresis donation were introduced. After their introduction, the number of 200-ml whole blood donation has been decreasing gradually, in contrast, the number of 400-ml donation has increased. In 1996, percentages of 400-ml and 200-ml donations were 44.1% and 35.7%, respectively. The number of apheresis donors has been relatively constant in these several years, consisting of 20.2% of donors in 1996. Majority of apheresis donors are donating platelet concentrates. Figure 2 shows a yearly profile of the platelet units supplied and composition of the unit number. It is obvious that platelet supply derived from 200-ml

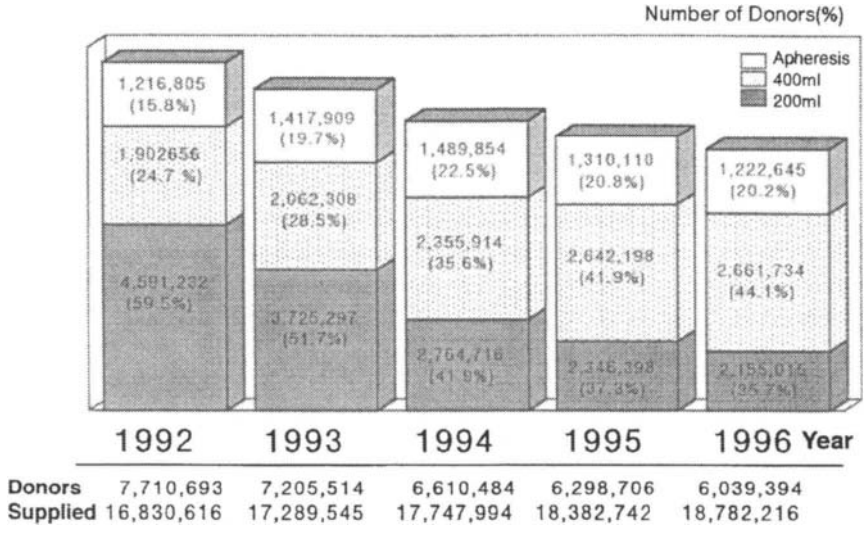

Fig. 1. Annual collection of blood.

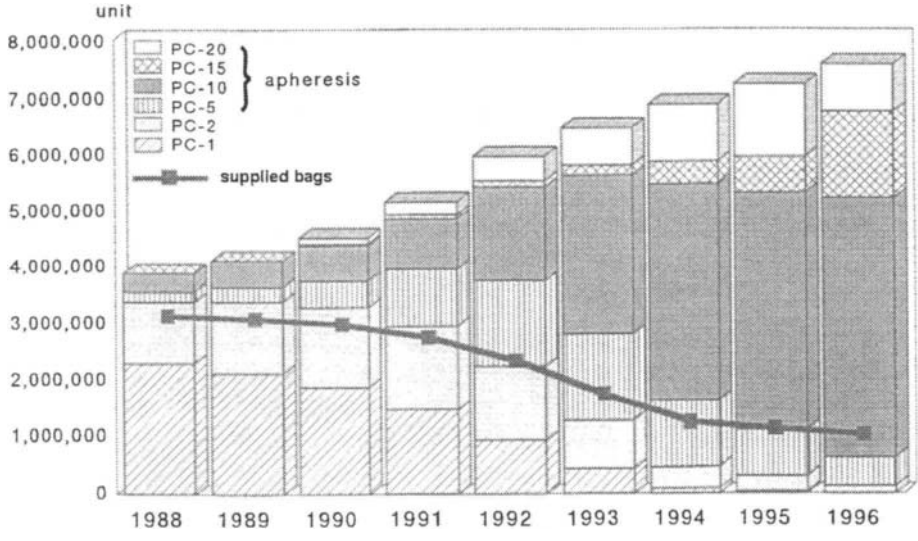

Fig. 2. Yearly profile of platelet supply classified by unit number of products.

and 400-ml donation (PC-1 and PC-2) has diminished yearly. At present the majority of platelet supply is from apheresis PC-10, PC-15 and PC-20. PC-1 or PC-2 is now rarely used in the pediatric cases. In contrast with the increasing number of platelet units, the donor number has decreased, because higher unit apheresis products occupy the majority.

The yearly supply of blood products is again shown in Fig. 3, including fresh frozen plasma. As already mentioned, the number of platelet supply markedly increased, but those of red cell concentrates and fresh frozen plasma show almost horizontal line. The usage of whole blood has decreased. Recent advance in chemotherapies and bone marrow transplantation in the oncology and hematology medicine, the necessity of platelet transfusion remarkably increased. In order to meet the demand, the introduction of apheresis protocol must have been necessary.

Plasma derivatives

This term consists of blood coagulation factors, serum albumin, and globulin. The use of plasma derivatives once increased around in 1975 in Japan, and in those periods the most of the products or their source plasma were then imported from other countries through commercial pharmaceutical manufactures. For this reason the percentage of the self-sufficiency of plasma products reduced to less than 10%. Thereafter, Japanese Red Cross Society established the plasma fractionation center to achieve self-sufficiency in plasma products in Japan. Recent self-sufficiency ratios of plasma products are listed in Table 1. Self-sufficiency for factor VIII is 66% in 1996,

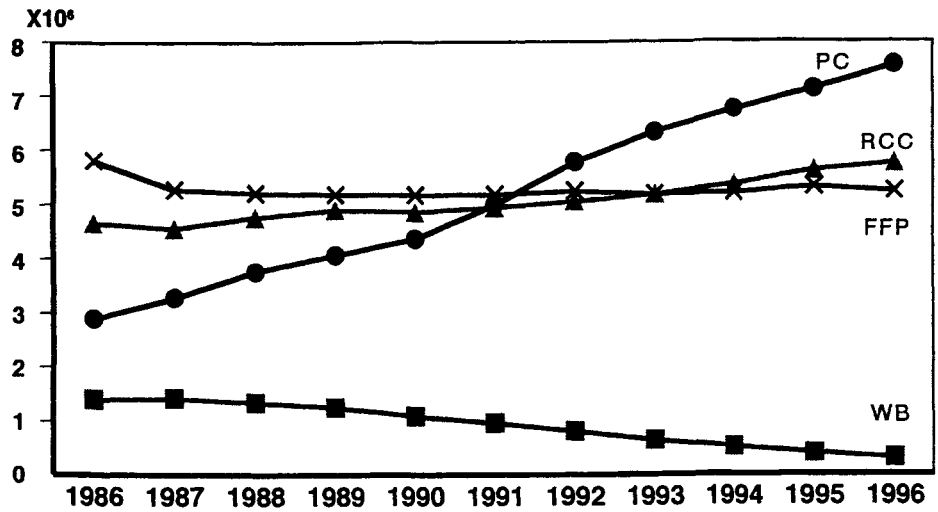

Fig. 3. Distribution of blood and blood components in units.

Table 1

Self-sufficiency of plasma products (1996)

Source plasma	74×10^4 l	Self-sufficiency
Factor VIII	$12{,}800 \times 10^4$ U	66%
Factor IX	250×10^4 U	76%
Albumin	18,800 Kg	25%
IV Globulin	1600 Kg	48%

but another 34% of factor VIII is supplied by recombinant factor VIII product also in Japan. The recent advance of molecular biological technology enables us to produce recombinant factor VIII. So at present the sum of plasma-derived factor VIII reaches 100% of the demand. During processing blood coagulation factors, factor VIII is first extracted from the source plasma, then factor IX is purified. Owing to this process, the problem of collecting source plasma for the production of factor IX will not separated exist. Unfortunately, both albumin and globulin still remain to be self-satisfied. Especially for albumin, its consumption in Japan consists of 20–30% of worldwide products. Some regulation must be introduced for the clinical usuage of albumin, but there are still attempts for clinicians to administer a large amount of albumin for edematous patients and patients with ascites.

Unused blood products

Due to the change of balance between the demand and supply of blood products and the presence of disqualified donors, a part of blood products will remain unused. The ratios are listed in Table 2. The ratios of outdated products from 400-ml and 200-ml

Table 2

Unused red blood cell products in Japan of 1996

Bags	400 ml	200 ml
Donations	2,660,000	2,110,000
Supplied Products	2,173,000	1,635,000
Unused Products	487,000	475,000
% of Donations	(18%)	(22%)
Disqualified Products	313,000	211,000
% of Donations	(12%)	(10%)
Outdated Products	174,000	264,000
% of Donations	(6%)	(12%)

Finally, total volume of outdated red cell products is estimated to be 84,680 l.

donations are 6% and 12%, respectively. Finally, total volume of outdated red cell products is estimated to be 84,680 l. Unless specially considered, these outdated products will be discarded. In order to maintain voluntary donations, it is desirable to apply these products for another usage. For example, from outdated red blood cells, we can recover hemoglobin and use it as a source material for hemoglobin-based substitutes. This is one of the reasons why we are attempting to develop red cell substitutes.

Transfusion-associated adverse effects and their prevention

The recent advancements in the donor screening tests mean that blood products have become increasingly safer in terms of transfusion-related viral transmission. Figure 4 shows the number and detail of transfusion-associated adverse effects reported to the Japanese Red Cross Central Blood Center. Now it has become mandatory for hospitals to report cases to the corresponding Red Cross Blood Center when adverse reactions occur. In 1996, about 700 cases of adverse reactions were reported. The majority of cases are non-hemolytic adverse reactions such as skin reactions, febrile reactions and anaphylaxis reactions. Seventy-four suspected cases of post-transfusion hepatitis were reported, but the precise analyses and comfirmative tests identified 2 cases to be highly probable of post-transfusion hepatitis. Another critical adverse reaction is graft-versus-host disease (GVHD). Sixty suspected GVHD cases were reported, 11 cases of which were confirmed to be definite GVHD cases by use of microsatellite-DNA examination. Figure 5 shows the number of reported transfusion-associated GVHD cases. Since information concerning GVHD has become common in the clinical field, the number of suspected cases has been increasingly

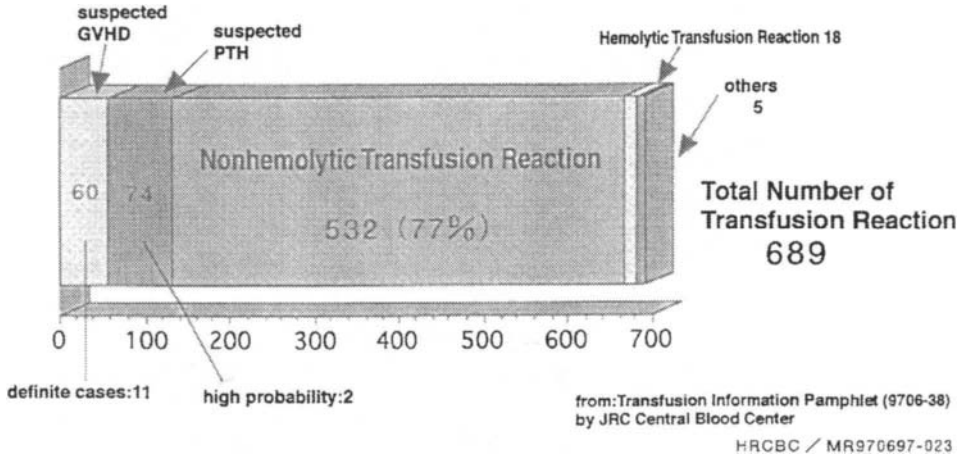

Fig. 4. The number of transfusion reactions in Japan (1996).

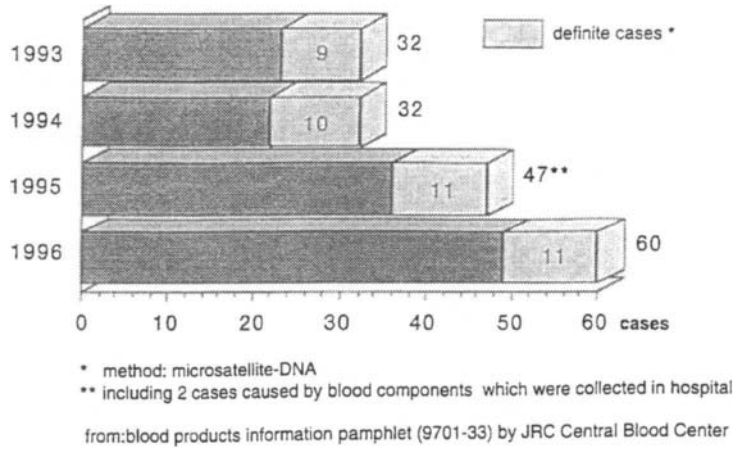

Fig. 5. The number of TA-GVHD cases reported to Japanese Red Cross Blood Centers.

reported. In contrast, however, around 10 cases still occur each year and the complete avoidance of GVHD has not been achieved yet [2].

Transfusion-associated viral transmission is still a critical problem. There still exists the risks of acquiring HIV and viral hepatitis. After the introduction of an anti-HIV test into the donor screening test in Japan in 1996, the first HIV infection case was reported early in 1997. Table 3 shows the probability of HIV transmission through the blood transfusion in the USA and Japan. Compared with the USA, the probability of transfusion-associated HIV transmission is fairly low, but still zero-risk has not been achieved. According to the statistical estimation, 1 bag out of 4.3 million bags may escape from the current screening test which can not detect donors in the window period of viral infection. The calculation indicates that 1 or 2 recipients will be transmitted HIV infection through blood transfusion annually in Japan. One remarkable point is the significantly higher risk in the metropolitan area in Japan. The HIV-positive ratio in that area is around 3 times higher than average ratio.

Table 3

Probability of HIV transmission by blood transfusion

	HIV positive rate of voluntary donors	Risk of HIV transmission by blood transfusion
USA (1994)	9.7 : 100,000 people	1 : 340,000 unit
JAPAN (1996)	0.76 : 100,000 people	1 : 4,320,000 bag
	(2.1 : 100,000 people)	(1 : 156,000 bag)

( ): The Metropolitan area.

Table 4

Estimated risk of major transfusion reaction

Major transfusion reactions	Reported transfusion reactions from hospitals to blood centers		
	No. (follow-up completed)	Cases of high-probability caused by transfusion	Incidence
Hepatitis B	69(22)	3	1/1,590,000
Hepatitis C	81(41)	1	1/4,770,000
HIV	1	1	1/12,000,000
HTLV-I	0	0	0
GVHD	139	30	1/160,000
Shock	239	—	1/20,000

From: Transfusion Information Pamphlet (9705-37), by JRC Central Blood Center.

The summary of major transfusion-associated adverse reactions and their frequencies is listed in Table 4. Since a great deal of effort has been made to reinforce the virus screening tests, the incidence of adverse reactions has decreased in Japan. With regard to hepatitis B, after the introduction of the combination analysis system using anti-HBc antibody and HBs antigen, the transfusion-associated hepatitis B has remarkably reduced in number and now the risk is about 1 case out of 1.5 million transfusion cases in Japan. A similar situation is also made possible for hepatitis C and the risk is about 1 case out of 4.7 million transfusion cases. There has been no report relating to transfusion-associated HTLV-1 transmission. In order to reduce the transfusion-associated risks to "zero", the development of blood substitutes should be necessary.

Future aspect of transfusion medicine

It is necessary to establish the safer transfusion or "zero" risk blood transfusion. The reduction of homologous blood transfusion is one of the main strategies and the prevailing of the autologous blood transfusion should also be the point. Again the efforts to reinforce the laboratory screening tests should be conducted further. The strategy to remove viruses or virus inactivation of the blood products should be further applied. In order to reduce the adverse reactions relating to contaminating leukocytes, the system to remove leukocytes or inactivate leukocytes must be prevailed in the clinical field. The development of blood substitutes will cover the functions of plasma, red cells and platelets. In addition, recent advancements in molecular biology will enable us to apply several hematopoietic cytokines to regulate the hematopoiesis. If we can expand hematopoietic stem cells, progenitor cells and mature functioning cells in a mega-culture system, it will become applicable to transfuse or transplant cultured cells into recipients (Fig. 6).

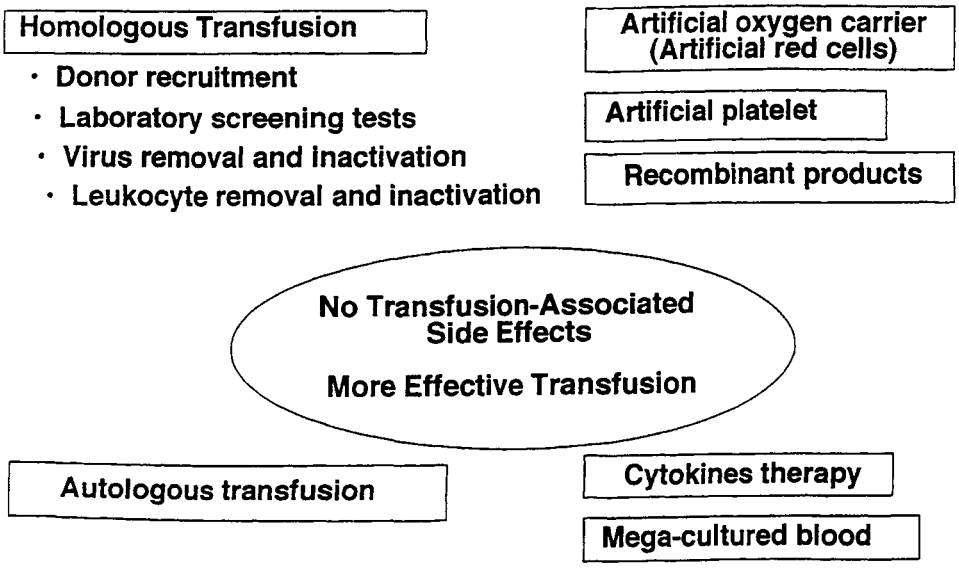

Fig. 6. Blood transfusion in the future.

Among them, the development of red cell substitutes have become near to practical usage. It is my opinion that the red cell substitutes will be used for the blood loss below 1200 ml and a further blood loss will be rescued by autologous blood or combined use of erythropoietin. The application protocol of artificial red cells should be discussed officially in early convenience [3].

Summary

The final goal of the blood program is to create a transfusion system which completes self-sufficiency by non-remunerated voluntary donors and supplys safer blood and blood products with "zero" transfusion-associated adverse reactions. To reach this goal, blood substitutes and substitute therapies can contribute a great deal in the near future.

References

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[2] S. Sekiguchi, M. Fujihara, Recent transfusion reaction and prevention. Journal of Clin. Anesth. (in Japanese), 21 (1997) 7–17.

[3] S. Sekiguchi, The impact of red cell substitutes on the blood service in Japan. Artif. Cells Blood Substit. Immobil. Biotechnol., 25 (1997) 53–60.